University of Alberta

Expression of SNARE Proteins, Potential Regulators of Exocytosis, in Human Basophils and Mast Cells

by

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Abstract

Mast cells and basophils, critically important granulocytes in allergic reactions and asthma, synthesize and secrete several proinflammatory mediators. I investigated expression of SNARE proteins using purified human basophils and the basophilic KU-812 and MC LAD-2, cell lines. Purified human blood basophils were obtained by negative immunomagnetic selection. Total RNA was extracted and subjected to RT-PCR. Western blot analysis and confocal microscopy were used to identify protein expression and distribution of distinct isoforms of SNARE complex. KU-812 were permeabilized with SLO, stimulated with Ca^{2+} and GTPγS and supernatants examined for surface CD63 (granular marker) expression and mediator release. Human basophils expressed mRNA for VAMP-1, -2, -3, -7, -8, SNAP-23, syntaxin-3, -4 and -6. KU-812 and LAD-2 shared a similar SNARE phenotype profile with human basophils. Syntaxin-4, -6 and SNAP-23 and VAMP-7,-8 were also identified by Western blot. Our findings support that basophil and MC mediator release involves SNARE complex assembly.

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I would like to dedicate this thesis to my husband,

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List of abbreviations

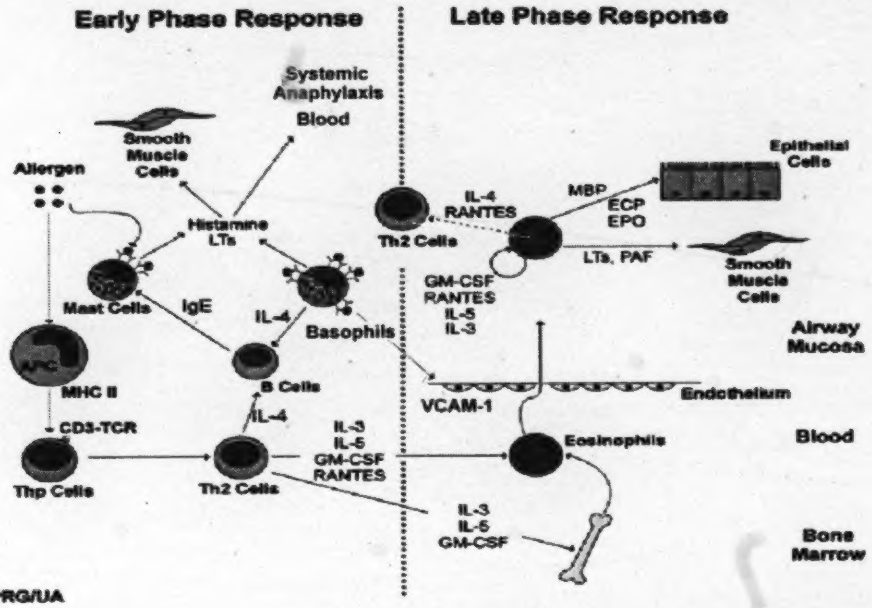
AND	Anaphylaxic degranulation
BAL	Bronchoalveolar lavage
BP	BODIPY-FL-conjugated antibody
β-Hex	β-hexosaminidase
CLC	Charcot-Leyden crystal
CLSM	Confocal laser scanning microscopy
CML	Chronic myelogeneous leukemia
CNT	Clostridial neurotoxin
DIC	Differential Interference Contrast
ELISA	Enzyme-Linked Immunosorbent Assay
EtBr	Ethidium bromide
FACS	Fluorescence-activated cell sorter
GVA	Granule-vesicle attachement
LAD-2	Laboratory of allergic disease-2 mast cell line
LT	Leukotriene
MACS	Magnetic activated cell sorter
MBP	Major basic protein
MC	Mast cells
MPO	Myeloperoxidase
NEM	N-ethylmaleimide
NSF	N-ethylmaleimide-sensitive factor
PAF	Platelet activating factor

PMD	Piecemeal degranulation
RR	Rhodamine Red-X-conjugated
RT-PCR	Reverse transcription-polymerase chain reaction
SLO	Streptolysin-*O*
SNAP	Soluble NSF attachment protein
SNAP-23	Synaptosome-associated protein of molecular weight 23 kDa
SNARE	SNAP receptor
TeNT	Tetanus neurotoxin
VAMP-7	Tetanus-insensitive VAMP (VAMP-7)
VAMP	Vesicle-associated membrane protein
WB	Western blot

1 CHAPTER

1.1 INTRODUCTION

Inflammation is an important feature in the pathogenesis of many diseases such as asthma, allergy and parasitic infections. It is a complex systemic response to foreign or internally produced substances. Inflammation is characterized by tissue infiltration with various cells including eosinophils, neutrophils, mast cells, basophils and macrophages (Figure 1.1). All cells undergo regulated exocytosis, a process of granule or vesicle fusion with plasma membrane and accompanied by release of granule/vesicle contents to the cell exterior. The release of mediators such as cytokines from inflammatory cells contributes to immune regulation, local inflammatory events, including tissue injury, followed by tissue remodeling. Despite its unifying importance to all inflammatory cell types, little is known about the precise molecular and intracellular mechanisms that regulate mobilization of secretory granules/vesicles leading ultimately to secretion of mediators from human basophils and mast cells. Understanding the complex molecular mechanisms involved in regulation of exocytosis may have potential therapeutic application by modulating of proinflammatory products and thus reducing or eliminating the clinical sequalae.


Early Phase Response
Late Phase Response
Systemic Anaphylaxis
Blood
Smooth Muscle Cells
Allergen
Histamine LTs
Mast Cells
IgE
IL-4
Basophils
APC
MHC II
CD3-TCR
B Cells
IL-4
Thp Cells
Th2 Cells
IL-3
IL-5
GM-CSF
RANTES
Th2 Cells
IL-4
RANTES
MBP
ECP
EPO
Epithelial Cells
LTs, PAF
Smooth Muscle Cells
GM-CSF
RANTES
IL-5
IL-3
Airway Mucosa
VCAM-1
Endothelium
Eosinophils
Blood
IL-3
IL-5
GM-CSF
Bone Marrow

RM/PRG/UA

Figure 1-1 Schematic diagram outlining function of inflammatory cells during allergic inflammation.

Following allergen exposure in sensitized individuals, allergen cross-linking of IgE bound to mast cell surfaces triggers rapid degranulation (exocytosis) and the release of mast cell mediators (histamine, leukotrienes (LTs), prostaglandins (PGs) and platelet activating factor (PAF). Mast cell mediators can activate other inflammatory cells to release several other mediators and contribute to pathological features of asthma such as mucus hypersecretion and smooth muscle contraction in small airways (bronchoconstriction). In chronic asthma a polarized T-cell response of the Th2 subtype predominates, which is characterized by high levels of IL-3, IL-4, IL-5 and GM-CSF. Secreted Th2 cytokines, particularly IL-5, stimulate eosinophilopoiesis in bone marrow. Human basophils can cause epithelial damage by releasing histamine. In addition, they may produce newly synthesized products such as IL-4 and IL-13 and lipid mediators (PGs and LTs), which contribute to pathological features of disease such as mucus hypersecretion and bronchoconstriction.

Modified from: Moqbel R.1999. Can. Respir. J. 6(5) 453-457

1.1.1 Human basophil and mast cell biology

1.1.1.1 Maturation and differentiation of human MC and basophils

Basophil development

In 1879, Paul Erhlich described metachromatic, granulated cells in the blood as an independent phenotype of leukocyte and distinguished them from tissue mast cells. Blood basophils have been referred to in the literature as Mastzellen, basophil granulocytes, and myelogenous mast cells. Although basophils were originally thought to be circulating mast cells, it is now well known mast cells and basophils are only distally related in hematopoiesis. They have a number of similarities, but also exhibit intriguing differences. An extensive review of this area was published by Hans Selye in 1965.

Because of the many similarities between mature basophils and mast cells, and since in healthy individuals the latter cell type are found only in tissue, it was initially proposed that basophils were circulating precursors of tissue mast cells. This concept implied that basophils infiltrate tissues thereby receiving an "unknown" signal(s) that causes them to differentiate into mast cells. There is presently compelling evidence to disprove this hypothesis. The average life span of basophils is a few days, with new cells being constantly produced from hematopoietic stem cells. The basophil lifespan more closely resembles that of eosinophils than that of mast cells, which are thought to have a turnover rate in tissue that is measured in months. Thus, *in vitro* cultures supplemented with supernatants derived from activated T lymphocytes showed that a common progenitor cell, colony-forming unit of eosinophil/basophil (CFU-Eo/B), is found in bone marrow, cord blood and, to a lesser extent, in peripheral blood, and can differentiate into mature basophils and eosinophils[1] (Figure 1.2).

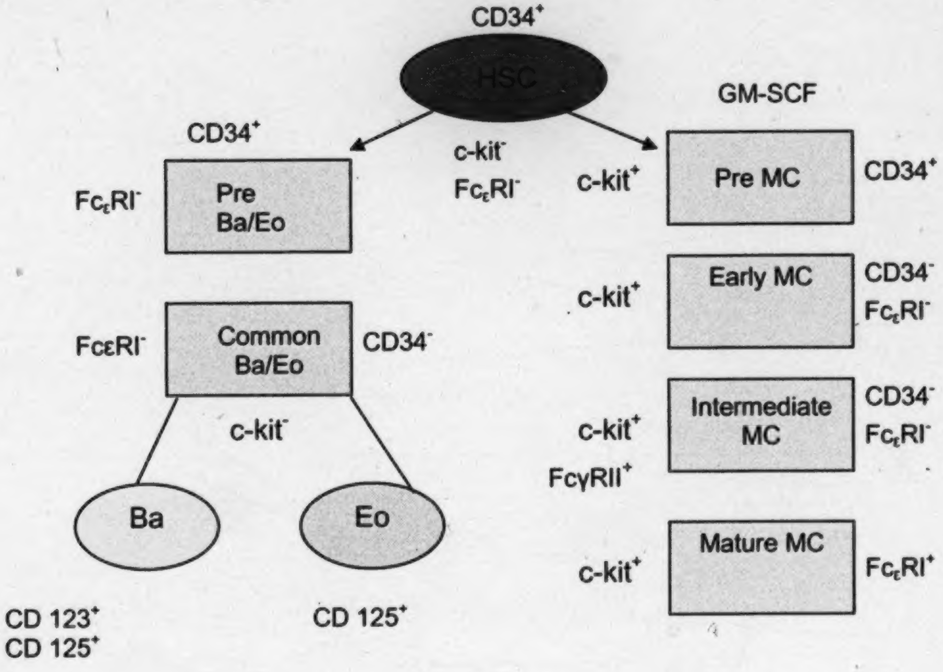

CD34$^+$
HSC
GM-SCF
CD34$^+$
c-kit$^-$
$Fc_{\varepsilon}RI^-$
c-kit$^+$
Pre MC
CD34$^+$
$Fc_{\varepsilon}RI^-$
Pre Ba/Eo
c-kit$^+$
Early MC
CD34$^-$
$Fc_{\varepsilon}RI^-$
$Fc\varepsilon RI^-$
Common Ba/Eo
CD34$^-$
c-kit$^-$
c-kit$^+$
Intermediate MC
CD34$^-$
$Fc_{\varepsilon}RI^-$
$Fc\gamma RII^+$
Ba
Eo
c-kit$^+$
Mature MC
$Fc_{\varepsilon}RI^+$
CD 123$^+$
CD 125$^+$
CD 125$^+$

Figure 1-2 Mast cell and basophil ontogeny.

An orderly sequence of differentiation is depicted, beginning with primitive CD 34+, $Fc_{\varepsilon}RI$-, c-kit- hematopoietic stem cell (HSC), and proceeding through various stages of commitment to either mast cells, basophils or eosinophils.

Modified from Basophilic Leukocytes: Mast cells and Basophils from Wintrobe's Clinical Hematology, tenth edition, Vol 1, pp 362-376 by A.D. Befus and J.A. Denburg.

Elevated numbers of CFU-Eo/B progenitors circulate in peripheral blood during asthma exacerbation. Numbers of human basophils increase by over 100 fold in bronchoalveolar lavage (BAL) and skin during allergic inflammation in atopic individuals[2-6]. It was noticed that the number of blood-borne metachromatic, histamine-producing granulocytes, basophils, are significantly increased in a number of myeloproliferative diseases, especially in chronic myelogeneous leukemia (CML)[7].

Over the last decade with the help of molecular cloning techniques a number of recombinant cytokines have been characterized that are important in differentiation of many cell types. For basophil development, pluripotential IL-3 or multi-colony-stimulating factor (CSF) has a profound effect in promoting differentiation of progenitors into cells that contain histamine, express high-affinity receptors for IgE, and resemble mature basophils, both morphologically and functionally[8]. However, the development of other leukocytes, including neutrophils, eosinophils, and monocytes, is also partially controlled by the actions of IL-3. After 2 weeks in culture supplemented with IL-3, there is a predominance of eosinophils, with some cells acquiring characteristics shared by both basophils and eosinophils[9]. The latter differentiation is likely the result of IL-5, which has an important role in maturation of progenitors into eosinophils. Unlike observations with murine mast cells, IL-3 does not support the development of human mast cells in these cultures. It appears that the *c-kit* ligand, or stem cell factor, plays a greater role in the development of mast cells from human $CD34^+$ precursors, whereas IL-3 is important for basophil development from these same precursors[10]. The cytokine, granulocyte-monocyte (GM)-CSF, which is an important granulocyte maturation cytokine, induces a disproportionate number of basophils after infusion, suggesting that

GM-CSF influences basophil development[11]. The cytokines thought to be involved in mast cell and human basophil differentiation are listed in Table 1.1.

Table 1-1 Cytokines involved in basophil and MC growth and differentiation.

GM-CSF	Basophil and eosinophil growth and differentiation; promotes in-vivo basophilia, eosinophilia and increase in circulating CFU-baso-/eo; basophil and eosinophil activation/survival
IL-3	Basophil and eosinophil growth and differentiation; basophil and eosinophil activation/survival; promotes in-vivo basophilia, eosinophilia; some mast cell differentiating activity.
IL-4	Binds to human mast cells; down regulates *c-kit* gene expression
IL-5	Basophil and eosinophil growth and differentiation; basophil and eosinophil activation/survival.
SCF	Basophil and mast cell growth and differentiation; may synergize with IL-3 in cord blood cultures

Modified from: Basophil and Mast Cell Lineages In Vivo and In Vitro, J.A. Denburg, Blood, Vol 79, No 4, 1992:pp846-860

The KU812 cell line was derived from a patient suffering from CML and has a number of characteristics of immature basophils. Many of these cells express receptors for IL-3 and have a tendency to differentiate more fully into basophil-like cells, synthesizing and storing substantial amounts of histamine and capable of binding IgE with high affinity[12,13]. IL-6, tumor necrosis factor (TNF)[14], IL-3[15] and IL-4[16] have been shown to induce differentiation in the KU-812 cells.

Mast cell development

Both mast cells and basophils arise from CD34$^+$ pluripotent bone marrow cells[17]. IL-3 is the major growth factor for human basophils[18, 19], but granulocyte-macrophage colony-stimulating factor (GM-CSF)[20], IL-5[21], and nerve growth factor[22] also promote basophil differentiation. IL-3, IL-5, and GM-CSF are also important in eosinophil differentiation. In contrast, human mast cells are deficient in receptors for IL-3[23].

Few, if any, MC develop when progenitor cells from bone marrow, cord blood, or fetal liver are exposed to human IL-3, IL-4, IL-10, or nerve growth factor, in the absence of SCF[24]. SCF, the ligand for Kit (CD117) (a product of the c-*kit* protooncogene), is the major growth factor for human MC[24, 25]. Two alternative splice variants account for different forms of SCF, one which is primarily membrane bound and the other which is primarily soluble after being released from the cell surface by proteolysis[26]. The fact that mast cells complete their differentiation in peripheral tissues rather than bone marrow suggests that SCF is available in these sites. Bone marrow stromal cells[27], like cells in peripheral tissues, produce both soluble or membrane forms of SCF. One possibility is that factors produced in the bone marrow environment divert SCF-treated progenitors to non-MC lineages. For example, IL-4 appears to prevent MC development from fetal liver, bone marrow, and peripheral blood progenitors[28], and may be present at higher concentrations in bone marrow than in peripheral tissue sites[29].

Human MC have been divided into two groups based on protease composition: MC_{TC} cells contain tryptase, chymase, cathepsin G, and carboxypeptidase, whereas MC_T cells contain only tryptase[30]. In normal-appearing pulmonary tissue, MC_T cells are the predominant mast cell type in the alveolar wall and in the epithelium of both the upper and the lower airway. MC_{TC} cells account for about 25% of the MC in bronchial subepithelium and 50% to 80% of the MC in nasal subepithelium. In the human asthmatic lung, MC numbers are increased and bronchial hyperresponsiveness correlates with MC number in both asthmatic adults and children[31]. MC numbers are also increased in aspiration pneumonia and post-viral infections[35]. Recent data have shown that an increase number of MC in muscle layers may contribute to airway constriction during asthma exacerbation[32]. In normal skin, mast cells reside in the dermis and are almost exclusively of the MC_{TC} type. In small bowel, MC_T cells tend to predominate in the mucosa, MC_{TC} in the submucosa. With allergic inflammation in the airway, increased numbers of mast cells, primarily of the MC_T phenotype, are found in the epithelium[33,34] whereas mast cell numbers and phenotype in subepithelial regions exhibit no apparent differences from normal tissue[35,36]. The importance of phenotypic differences among MC suggests the selective role of those cells in inflammation.

What causes mast cells to localize to certain tissues and to develop as MC_T or MC_{TC} cells remains unknown. Progenitors may localize to tissue sites where soluble SCF is being generated. RANTES, an eosinophilotactic chemokine, also appears to be chemotactic for human mast cells[37]. Once mast cells arrive in tissues, their location may be influenced by their adhesion molecules. Human mast cells express β_1 (CD29) and β_3 (CD61) integrins, but few, if any, of the β_2 (CD18) integrins. The predominant β_1 integrins include those with α_4 (CD49d) or α_5 (CD49e). α_v/β_3 (CD51/CD61) is the predominant β_3 integrin on the surface of MC. These integrins recognize extracellular matrix proteins such as vitronectin, fibronectin, and laminin, as well as vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells and the CD44 receptor for hyaluronan.

In contrast to MC, human basophils, do not localize to tissues unless recruited into an inflamed site. Factors known to be chemotactic for human basophils include IL-3, IL-5, GM-CSF, C5a, IL-8, platelet activating factor (PAF)[38],[39]. These factors also recruit eosinophils and, in some cases, neutrophils. Thus, whereas MC recruitment to tissues is a component of the natural development of these cells, basophil recruitment occurs in response to inflammation.

1.1.1.2 De-novo synthesized and stored mediators in human basophils and mast cells

Mast cells and basophils are storehouses of inflammatory mediators that can be released by IgE-mediated and other stimuli (Figure1.3). Activation of mast cells and basophils results in the release of mediators responsible for much of the clinical response observed during immediate-type hypersensitivity reactions. Degranulation releases preformed mediators that are stored inside secretory granules and occurs within minutes after the cells are stimulated. Metabolites of arachidonic acid are also generated after stimulation and are secreted over 5-30 minutes.

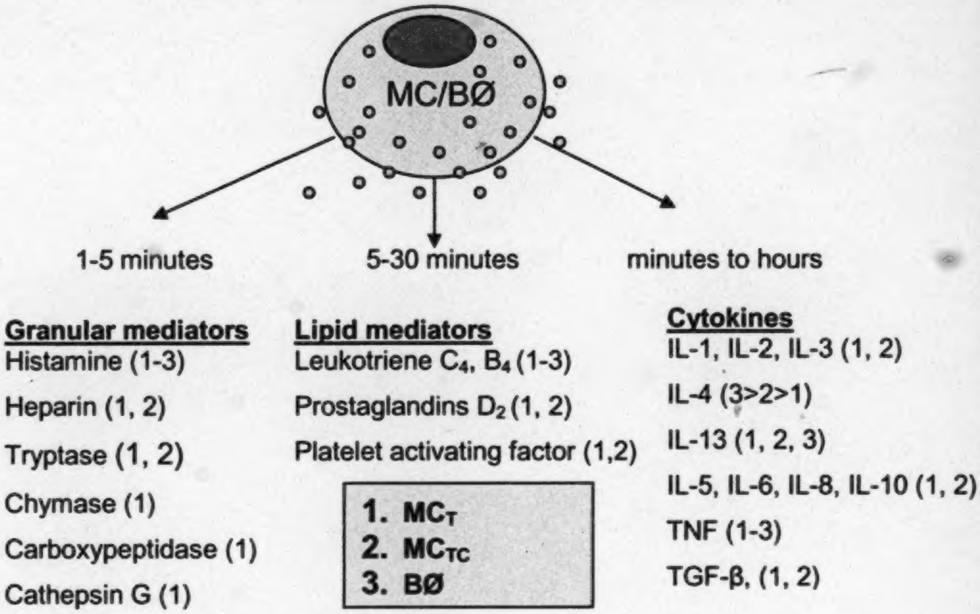

MC/BØ
1-5 minutes
5-30 minutes
minutes to hours
Granular mediators
Histamine (1-3)
Heparin (1, 2)
Tryptase (1, 2)
Chymase (1)
Carboxypeptidase (1)
Cathepsin G (1)
Lipid mediators
Leukotriene C4, B4 (1-3)
Prostaglandins D2 (1, 2)
Platelet activating factor (1,2)
1. MCT
2. MCTC
3. BØ
Cytokines
IL-1, IL-2, IL-3 (1, 2)
IL-4 (3>2>1)
IL-13 (1, 2, 3)
IL-5, IL-6, IL-8, IL-10 (1, 2)
TNF (1-3)
TGF-β, (1, 2)

Figure 1-3 Mediators released by mast cells and basophils.

Human basophils and MC after activation by an immunoglobulin E-mediated process. Within *parentheses* next to each mediator is shown whether MC_T, MC_{TC}, or basophils are the major source.

Table 1-2 Selected Mediators of Human Basophils and Mast Cells.

(*Note ; NK = not known)

Mediator	Cell Type	
	Mast Cells	Basophils
Histamine	+	+
Platelet activating factor	+	+
Nitric Oxide	+	NK*
Proteoglycans	Heparin, Chondroitin sulfates	Heparin, Chondroitin sulfates
Arachidonic acid metabolites	LTB_4, LTC_4, PGD_2, PGF_2, thromboxane A_4	LTB_4, LTC_4
Cytokines/Chemokines	IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, TNF, TGF-β, IFN-γ, GM-SCF	IL-4, IL-13, MIP-1α, TNF
Proteases	Neutral proteases (chymase and/or tryptase)	Neutral proteases with bradykinin-generating activity

Although variable amounts of different cytokines may be present at the time of stimulation, more typically they are synthesized after stimulation, leading to their secretion over several hours.

While antigen cross-linking of IgE has an important role in basophil activation, a growing list of diverse stimuli can also induce mediator release from these cells. Other studies show that the basophils isolated from asthmatics, while demonstrating releasability in response to a number of stimuli, also show increased spontaneous release of mediators. It is important to note, however, that many of these same secretogogues have been shown to have little or no activity on mast cells[40, 41], suggesting that the basophil is far more excitable in nature. For example, C5a is a highly potent inducer of histamine release from basophils following binding to the C5a receptor, CD88[42]. In contrast, most human MC subtypes do not express CD88 and are unresponsive to C5a except skin MC. Moreover, unlike basophils, mature MC, including those of the skin, also do not express receptors for IL-3 and GM-CSF[43]. This clearly suggests that, compared with MC, basophils are more sensitive to the effect of immunomodulatory cytokines and proinflammatory factors.

The production of cytokines and chemokines is a common occurrence during different immune reactions, such as allergic inflammation[44]. Many of these have multiple functions acting on many different cell types. Therefore, it seems appropriate to state that cells that secrete cytokines in sites of allergic inflammation have important roles in development, progression, and outcome of the responses.

Basophils secrete both preformed and newly-synthesized mediators following activation with a wide range of stimuli. Histamine and leukotriene (LT) C_4 have been studied extensively for many decades and have generally been thought to be the primary proinflammatory substances released by human basophils[45]. Upon activation by a variety of stimuli (fMLP, anti-IgE, calcium ionophore, C5a and substance P)[46], basophils also synthesize and release the arachidonic acid metabolite, LTC_4[47]. Several phospholipids, such as phosphatidylcholine and phosphatidylinositol, serve as the source of arachidonic acid. The phospholipids are cleaved directly by phospholipase A_2, or by the sequential action of phospholipase C and diglyceride lipase. A calcium-sensitive 5-lipoxygenase then metabolizes arachidonic acid into LTC_4 by glutathione transferase. Further metabolism of LTC_4 into LTD_4 and LTE_4 does not occur in pure basophil suspensions, but apparently depends on the presence of other leukocytes. Unlike mast cells, there is little evidence for the release of prostaglandins in response to activation, suggesting that cyclooxygenase enzyme activity is not as common in basophils as is the lipoxygenase pathway.

Approximately 10 to 100 fg/basophil of LTC_4 is formed and released upon activation, some 100-fold less than the amount of histamine secreted by these cells[48]. Not withstanding, on a molar basis LTC_4 is 100 to 6,000 times more potent in contracting smooth muscle[49]. Prolonged wheal and flare reactions and mucus secretion are also stimulated, indicating that LTC_4 is likely a participant in airway events associated with an asthmatic attack[49]. In other studies, leukotrienes affect the immune response of many cell types, acting as chemotactic factors in addition to promoting the production or inhibition of various cytokines.

Graham et al. in 1952 demonstrated the presence of histamine in blood basophils. This study showed that blood basophils contain about 50 times more histamine than any other blood cell. However, blood basophils contain about 1 pg of histamine per cell, i.e. about 1/20 that found in tissue mast cells[50]. Since then an extensive research has been done on ultrastructural characteristics of human basophils[51, 52]. It has become clear that: (1) basophils represent the single cellular source of peripheral blood histamine in humans and (2) a wide variety of secretogogues induce histamine secretion from basophils.

Histamine is synthesized from L-histidine by histidine decarboxylase and then stored in membrane-bound cytoplasmic secretory granules[53] in a complex with highly sulfated proteoglycans (mostly chrondroitin). Histamine is a potent smooth muscle spasmogen that can also cause an increase in tissue fluid by dilating terminal arterioles and constricting postcapillary venules[54]. Histamine can also downregulate several T-cell-mediated immune responses by binding to H_2 receptors and causing elevations in intracellular cyclic adenosine monophosphate (cAMP)[55]. It seems likely that this inhibitory activity is directed at T-helper type 1 (TH1)-like functions, particularly since basophils are increasingly thought to promote TH2-like responses through the secretion of cytokines (Figure 1.1).

There is now substantial evidence that basophils are capable of generating high levels IL-4 mRNA and protein upon activation[56,57] and these cells secrete other cytokines important in allergic inflammation, such as IL-13 (Fig.1.3, Tab.1.2). IL-4 has many regulatory roles in the allergic response. Given that activated basophils are a significant source of this cytokine, these cells have an important role in the pathogenesis of allergic disease.

Unlike the release of histamine or LTC_4, which occurs upon degranulation or within minutes following stimulation, generation of IL-4 protein by basophils occurs *de novo* upon activation, with only a small amount of IL-4 stored in cytoplasmic granules. *In vitro* studies show that after IgE-dependent stimulation, IL-4 protein is first detectable within 5-10 min[57], with levels peaking by 4 to 6 h[58,59]. In these same cultures, however, histamine and LTC_4 are almost completely released 15 to 20 min following activation.

With the availability of ultrasensitive enzyme-linked immunosorbent assay (ELISA) kits to detect protein levels of less than 1 pg/ml, it is now possible to measure IL-4 protein secreted by the small number of basophils found in approximately 1 ml of blood, or the amount of blood commonly used for histamine analysis. There is a correlation between the amount of newly synthesized IL-4 protein and the magnitude of histamine released, with donors whose basophils show a high percentage of histamine release also secreting the highest levels of IL-4. The highest levels of newly synthesized IL-4, however, are secreted in response to concentrations of anti-IgE antibody (or antigen) that are 10-fold less than the amount necessary for the highest levels of histamine release. This observation, along with more recent findings[60], indicates that intracellular mechanisms controlling histamine and IL-4 secretion diverge at some point after the formation of antigen-receptor complexes.

Mediators produced from MC have classically been divided into three categories: preformed mediators, newly synthesized lipid mediators and cytokines. Preformed mediators are packaged within secretory granules. At activation, granule contents are released into the intracellular environment within minutes. Principal granule constituents include histamine, serine proteases, carboxypeptidase A and proteoglycans (heparin and chondroitin sulfate E). Human MC contains approximately 2 pg of histamine per cell. Histamine has effects on smooth muscle (contraction), endothelial cells, nerve endings and mucous secretion. Heparin and chondroitin sulfate proteoglycans are believed to aid storage of preformed molecules, which dissociate from these proteoglycans at variable rates in physiologic buffer solutions. Heparin itself is capable of anticoagulation through binding to antithrombin 3.

Much of the protein in MC granules is made up of four neutral proteases: tryptase, chymase, carboxypeptidase and cathepsin G. The function of tryptase in vivo is unknown, but in vitro it can cleave C3 and C3a, activate fibroblasts and promote accumulation of inflammatory cells. Both α and β tryptase have been described and it has been said that α form is constitutively expressed, whereas β form is released during degranulation.

The major lipid mediators synthesized by MC include prostaglandin D_2 (PGD_2) the major cyclooxygenase product. PGD_2 is produced by MC, but was not detectable in human basophils. MC synthesize the lipoxygenase product LTC_4: extracellular processing of LTC_4 yields the active metabolites LTD_4 and LTE_4. Skin MC produce more PGD_2 than LTC_4, whereas the opposite is true of MC from the lung. PGD_2, LTC_4, LTD_4 and LTE_4 are all bronchoconstrictors.

Human MC are capable of synthesizing and secreting an array of cytokines (Table 1.2). IL-4 produced by MC associated with Th2 cell differentiation and IgE synthesis; IL-3, GM-CSF and IL-5 are critical for eosinophil development and survival[61].

The summary table of major differences between MC and human basophils is shown in the Appendix (Table A-1).

1.1.1.3 Human basophil: role in allergic inflammation and beyond

1.1.1.3.1 Basophils in allergic inflammation

Recent studies have demonstrated the appearance of basophils in tissues following a local allergic reaction[62]. Evidence exists that basophils increase dramatically in the airways of patients who die of severe asthma, accumulating in numbers similar to those of mast cells[63]. Further, additional biopsy specimens taken from asthmatic individuals reveal a correlation between basophil number and the severity of the asthmatic symptoms[64]. Although these observations strongly implicate the participation of basophils in allergic lesions, the exact nature of their role in allergic disease is not fully understood.

Studies on the clinically relevant late-phase reaction to antigen challenge best support the involvement of basophils in chronic allergic inflammation. Although the cellular infiltrates during these late reactions show a predominance of eosinophils, the early work by Okuda and Otsuka[65] clearly showed that basophils also migrate into the nasal mucosa. In fact, the nasal tissue of rhinitic patients provides a convenient test site for the experimental *in vivo* challenge model of the allergic late reaction. An increased number of human basophils was observed after nasal provocation and during pollen challenge, which showed a decrease in basophils numbers following a course of immunotherapy or during the off-season of pollinosis [65]. Basophils numbers in the nose positively correlated with nasal symptoms, tissue eosinophilia, and nasal provocative reactions. The nasal secretion fluid contains measurable mediators of the allergic reaction and cells that have migrated into the nasal lumen by way of the nasal mucosa[66]. Challenge of atopic patients with the appropriate antigen leads to the rapid appearance of mediators such as histamine, prostaglandin (PG) D_2, LTC_4, together with the usual symptoms of the allergic reaction, such as sneezing and nasal congestion. In approximately 50% of the patients, this initial response was found to subside within an hour, only to return 5 to 12 h later (late phase reaction). Both symptoms and mediators would return back with the notable exception of PGD_2. This mediator would reappear only after a second antigen challenge. Previous studies indicated that mast cells but not basophils release PGD_2 in an IgE-mediated reaction, and it was thus concluded that basophils contributed to the late reaction. Also, sufficient numbers of these cells have been collected and shown to possess functional characteristics consistent with basophils, including histamine content and sensitivity to both anti-IgE and formylated methyl leucyl phenylalanine (fMLP) identical to peripheral blood basophils[67]. Other evidence supporting the role of basophils during the late phase reaction came from administration of oral or a short course of topical steroids prior to experimental challenge. The early reaction (mast cell response) was not significantly altered, but the late phase (basophil response) was nearly abolished[3].

This *in vivo* model of allergic reactions was confirmed by replicating the same set of experiments in skin and lung[68]. For skin studies, a blister was made and the epidermis removed and replaced with a chamber into which allergens were instilled and fluids removed for examination. In lung studies, following instillation of ragweed antigen directly into an airway segment of allergic asthmatic subjects, fluids were removed for study. In both skin and lung studies, many of the above features of the reactions observed in the nose have been replicated, including the appearance of basophils in the late reaction.

There is substantial evidence to suggest that the late phase reaction is characteristic of conditions in the lung tissue of asthmatic patients, suggesting that basophils may play an important role in the pathogenesis of asthma related allergic reactions. This belief may gain further support now that basophils have recently been shown to secrete large quantities of the immunoregulatory cytokine, IL-4 and additional cytokines important in allergic inflammation, including IL-13[69]. During the late-phase reactions, IL-4 and IL-5[44] were implicated in the pathogenesis of allergic inflammation, including that associated with asthma. In fact, the kinetics of IL-4 secretion by basophils is consistent with the timing where these cells first appear in tissue sites during a late-phase response. Since this IL-4 release is considerably faster than the 12 to 16 h reported for the production of this cytokine by antigen-stimulated T lymphocytes[70], basophils may have a more significant role in initiating the late-phase reaction. The adherence and selective recruitment of eosinophils are partially controlled by the actions of IL-4 on endothelium[71]. As such the accumulation of eosinophils at sites of allergic inflammation may also been facilitated by human basophil activation. Finally, a number of studies have shown that the development of TH2 and TH1 $CD4^+$ lymphocytes is regulated by the actions of IL-4 and interferon γ (IFNγ) on naive cells, respectively[72]. Thus, basophil-derived IL-4 may upregulate the development of TH2 lymphocytes, because there is a predominance of activated cells of this phenotype at sites of allergic inflammation. Therefore, it is possible that the secretion of IL-4 (and likely other cytokines such as IL-13) from basophils might help amplify and maintain allergic inflammation.

Recent evidence has implicated the cytokine IL-13, produced in large concentrations by activated basophils, as a key mediator of allergic asthma[73]. In humans, IL-13 is secreted following local allergen challenge in subjects with mild atopic asthma[74]. Use of IL-13R-Fc fusion protein that specifically neutralizes IL-13, indicated that this cytokine is necessary and sufficient for establishment of an asthmatic phenotype in a murine model[75]. Administration of soluble recombinant IL-13 was effective in inducing airway hyperresponsiveness and in generating a significant recruitment of eosinophils to the lung, as shown by their appearance in BAL[75]. In vitro, IL-13 induces recruitment and prolongs the survival of eosinophils[76], up-regulates VCAM-1 expression on vascular endothelium[77], and is a very potent inducer of eotaxin in airway epithelial cells[78]. The importance of IL-13 in the pathogenesis of asthma is also corroborated by a recent study using transgenic mice that release IL-13 selectively in the lung[79]. The authors of this study found that transgenic mice, in the absence of a specific sensitization, developed a phenotype very similar to the asthmatic phenotype, including eosinophil infiltration, up-regulation of eotaxin, mucus hypersecretion by goblet cells, subepithelial airway fibrosis, and airway hyperresponsiveness to methacholine[79].

Numerous studies have also demonstrated that basophils participate in cutaneous basophil hypersensitivity reactions in humans. For example, basophils can be found in significant numbers in allergic contact dermatitis reactions. Other reactions normally associated with cellular immunity, skin allograft[80] and tumor rejection, viral hypersensitivity[81], and Crohn's disease, and also show association with a high numbers of basophils. The best described *in vivo* model of this reaction, the delayed contact skin hypersensitivity to dinitrochlorobenzene in humans, demonstrates that basophils are often the only granulocyte to infiltrate the skin during the first 3 days of the reaction[82]. It is not until later in time that a few eosinophils and neutrophils appear. It has been difficult to understand the mechanism of basophil infiltration occurring in these reactions and to ascertain whether basophils play an important role.

Recently, it has been reported that the surface marker CD63 is expressed with high density on activated basophils yet only weakly on resting cells[83-85]. The CD63 glycoprotein is present on the membrane of cytoplasmic granules, is expressed only to an extent of less than 5% on the outside membrane of resting basophils, but with high density on activated basophils after the fusion of these granules with the cytoplasmic membrane[86, 87]. We used CD63 expression as a granule marker to detect co-localization with the SNARE proteins. In a separate study, we used CD63 as surface marker for degranulation upon introduction SNARE-specific antibodies to inhibit the release of mediator from human basophils.

1.1.1.3.2 Basophils in host defense

Granulocytes are part of the innate immune system, which is a first line of defence against invading pathogens. In animals experimentally infected with nematodes, basophils have been shown to proliferate in the bone marrow and to increase up to 50-fold in the circulation[88-89] and to accumulate and degranulate at the site of infection. In rats infected with *Nippostrongylus brasiliensis*, the period of expulsion coincides with the peak of blood basophilia[89], suggesting involvement of basophils in immunity (Figure 1.4). In humans infected with the hookworm *Necator americanus*, basophil levels are increased in peripheral blood and are the only cell population responsive to worm removal after treatment with anthelminthics[90] This finding, apart from suggesting that basophils be used as indicators of successful treatment and worm removal, also suggests that hookworms induce basophilia. In humans it was noted that the rejection of schistosome worms and ectoparasitic arthropods largely depend on basophils. Tick rejection in guinea pig skin is eliminated if the animals are first treated with a specific antibasophil antibody that specifically ablations bone marrow, peripheral blood and infiltrating basophils[91].

Particular interest has been generated by studies showing the activation of N-formyl-peptide receptor on basophils by *Helicobacter pylori* antigens[92], HIV-1 envelope pg41 peptides[93] and urokinase[94] which cause basophil chemotaxis. Bacterial peptidoglycans can activate basophils by stimulating TLR2 (Toll-like receptor 2), leading to IL-4 and IL-13 release, and further enhance basophil mediator secretion caused by IgE-dependent triggering[95]. Several plant lectins (concanavalin A) cause basophil activation with IL-4 secretion due to IgE crosslinking caused by lectin binding[96], providing further evidence of basophil capacity to support Th2 responses.

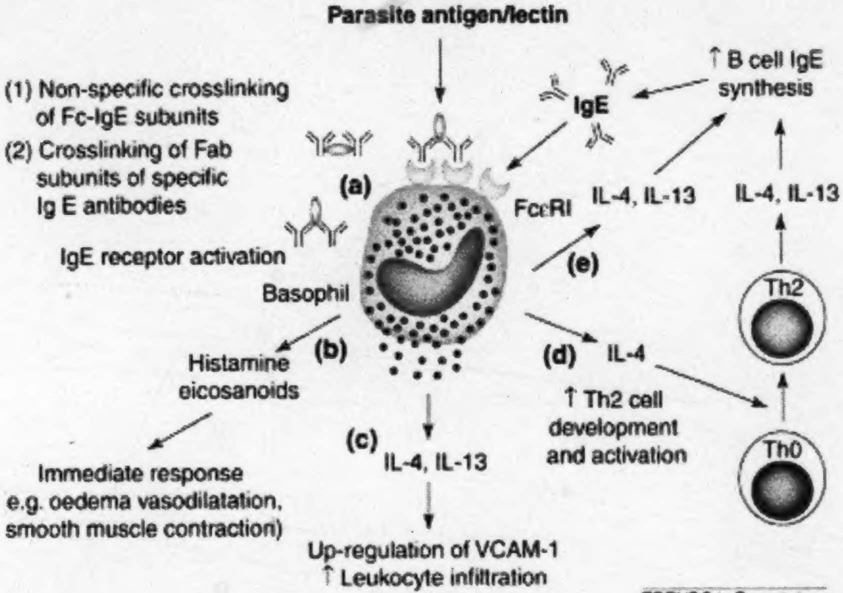

Parasite antigen/lectin
(1) Non-specific crosslinking of Fc-IgE subunits
(2) Crosslinking of Fab subunits of specific Ig E antibodies
IgE receptor activation
(a)
IgE
↑ B cell IgE synthesis
FcεRI
IL-4, IL-13
IL-4, IL-13
(e)
Th2
Basophil
(b)
Histamine eicosanoids
(d)
IL-4
↑ Th2 cell development and activation
Th0
(c)
IL-4, IL-13
Immediate response e.g. oedema vasodilatation, smooth muscle contraction)
Up-regulation of VCAM-1
↑ Leukocyte infiltration

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Figure 1-4 Basophils can influence immune responses.

(a) Antigen specific (e.g. parasite antigens or allergens) and non-antigen specific stimuli (e.g. lectins specific for IgE carbohydrate side chains, superallergens) crosslink the high-affinity receptor for IgE (FcεRI), resulting in basophil degranulation. (b) Release of preformed (histamine) or *de novo* synthesized substances (eicosanoids) induces immediate effects such as vasodilatation and smooth muscle constriction. Within hours of degranulation, the basophils release large amounts of IL-4 and IL-13, which might play a pivotal role in biasing the emerging immune response to the Th2 phenotype. (c) Increased expression of the adhesion molecule VCAM-1 on the endothelial cell surface by the basophil-derived cytokines IL-4 and IL-13 augments inflammatory cell extravasation and infiltration into the tissues. Basophil-derived cytokines can also play a central role in skewing T-cell differentiation to the Th2 phenotype (d) as well as directly switch ongoing antibody production to the IgE isotype (e).

From Falcone FH et al. Do basophils play a role in immunity against parasites? Trends Parasitol. 2001 Mar;17(3):126-9.

1.1.1.3.3 Basophils in hematopoietic malignancies

The prototypical hematopoietic malignancies associated with basophil and mast cell lineage and their dysregulation include chronic myelogeneous leukemia and related myeloproliferative disorders as well as systemic mastocytosis. Hematopoietic malignancies affect the myeloid lineage that produces granulocytes and macrophages. Basophilia or basophil crisis heralding terminal blast crisis in CML has long been known[97-98]. The number of basophils in circulation is a useful predictor of accelerated phase of CML and is a significant prognostic and staging factor. In addition, hyperhistaminemia is an important marker in CML and myeloproliferative disorders, indicating increased number or turnover of basophils[99,100] and mediator release. *In vitro* observations indicated that indices of increased basophil growth and differentiation are also poor prognostic indicators in CML and related myeloproliferative disorders[101]. All these factors may relate to fundamental aberrations in chromosomes that reflect the clinical and pathological picture and atypical forms of leukemia[102,103,104]. Numerous studies have identified an inversion of chromosome 16 associated with atypical eosinophils and basophils; t (6,9) chromosomal translocation associated with basophilia and leukemia; trisomy 21 (Down syndrome) associated with increased number of basophils[105]. The increased basophil numbers are regularly accompanied by marrow eosinophilia that may reflect abnormal differentiation of leukemic cells with the capacity to differentiate into both eosinophilic and basophilic pathways. The precise relationship between chromosomal aberrations and atypical differentiation of basophils or their clinical implication has not been delineated.

1.1.2 Exocytosis of stored mediators from basophils

1.1.2.1 Patterns of exocytosis in human basophils

All inflammatory cells undergo exocytosis, a process of granule or vesicle docking and fusion with the plasma membrane, accompanied by release of granule/vesicle contents to the surrounding extracellular environment. Exocytosis occurs by a highly regulated series of events. These include mobilization or translocation of the vesicle/granule to the cell periphery, tethering of the granule to the plasma membrane, and docking between the vesicle/granule and plasma membrane, which is critically important for membrane fusion and mediator release.[106]

Despite the importance of exocytosis in mast cells and basophils, little is known about the precise molecular and intracellular mechanisms that regulate the downstream events from cellular activation leading to mobilization of secretory granules, their docking and ultimately, secretion of mediators. Granule mobilization is defined as a recruitment of signaling molecules and specific motor proteins to the granule membrane and the subsequent migration of granules along microtubules and actin[107]. Docking collectively refers to the physical interaction of the outer leaflet of the granule membrane with the inner leaflet of the plasma membrane. Membrane traffic is fundamental to the integrity of inflammatory cells and is regulated by conserved protein families, particularly soluble *N*-ethylmaleimide–sensitive factor attachment protein receptors (SNAREs)[108].

The release of granule-derived proteins from human basophils and mast cells to the extracellular space has been referred to as degranulation. Extensive work has been done by Dvorak and colleagues to characterize the unique pattern of secretion and degranulation exhibited by tissue basophils in tumor lesions[109] and contact allergy skin lesions[110]. She outlined two main patterns of degranulation in human basophils: anaphylactic and piecemeal degranulation (PMD). In other cell types, such as eosinophils[111] several types of degranulation were proposed that give a conceptual overview of mediator release. Patch-clamp analyses have supported the notion that human basophils do exhibit compound and piecemeal modes of secretion[112].

There are four main types of degranulation described in human mast cells and basophils (Figure 1.5). **(1) Compound** exocytosis is characterized by granule-granule fusions within the cell followed by a single large exocytotic event resulting from granule membrane fusion with the plasma membrane. Classical exocytosis utilised the same type of degranulation, but it occurs by single granule fusions with the plasma membrane. **(2) Piecemeal degranulation (PMD)** involves the mobilization and secretion of small vesicels independent of large granule events and is implicated in the selective release of stored mediators. **(3) Anaphylaxic degranulation (ADN)** characterized by explosive and rapid granular extrusion with the subsequent slow recovery. **(4) Cytolytic degranulation** is defined as deposition of free floating granules and protein contents with the subsequent loss of cell integrity. Mediator release via cytolysis, although physiologically relevant, is not considered a form of exocytosis.

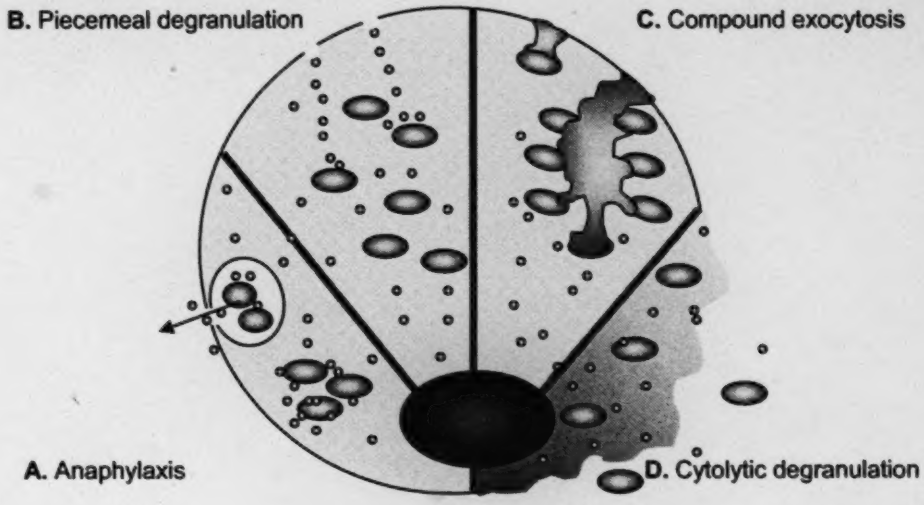

Figure 1-5 Patterns of degranulation in human basophils.

There are four main types of degranulation described in human mast cells and basophils. **(A) Anaphylaxic degranulation (AND)** characterized by explosive and rapid granular extrusion with the subsequent slow recovery of granules and small vesicles. **(B) Piecemeal degranulation (PMD)** involves the mobilization and secretion of small vesicles independent of large granule events and is implicated in the selective release of stored mediators. **(C) Compound exocytosis** is characterized by granule-granule fusions within the cytoplasm followed by a single large exocytotic event resulting from granule membrane fusion with the plasma membrane. **(D) Cytolytic (necrotic) degranulation** is defined as extrusion of free floating granules and protein contents with the subsequent loss of cell integrity. Mediator release via cytolysis, although physiologically relevant, is not considered a form of exocytosis.

Modified from Ann M. Dvorak, Ultrastructural analysis of the development of human basophils and mast cells in vitro ,Int J Lab Re s25:7-24, 1995

1.1.2.1.1 Anaphylactic degranulation

Anaphylactic degranulation (AND) is a general term used to describe the rapid regulated secretory events of which mast cells and basophils are capable[113]. AND occurs mainly following stimulation through the IgE receptor or following other stimuli such as fragments of the complement cascade. Prominent changes can be seen during AND: cytoplasmic filaments mobilized around granules, large pore formed thereby extruding vesicles and granules (Figure 1.5.A). AND corresponds to explosive granular extrusion by exocytosis, a regulated secretory process common to all secretory cells. This is an event in human basophils which is completed within minutes of stimulation. Upon appropriate stimulation human basophils can secrete also Charcot-Leyden crystal (CLC). CLC has been designated as galectin-10[114] . Eosinophils and basophils are so far the only known sources of CLC protein and dense concentric membranes through multiple pores in the cell membrane[115]. After completing the degranulation phase in ADN, basophils can remain viable and may slowly recover.

1.1.2.1.2 Piecemeal degranulation

Mature basophils are characterized by large number of cytoplasmic vesicles, some of which are associated with granules. PMD is a term introduced to explain the ultrastructural finding of partially or completely empty cytoplasmic granules (Figure 1.5.B), in the absence of granule fusions to the plasma membrane. Cytoplasmic granules that exhibited loss of contents were observed, and were associated with the characteristic presence of numerous small cytoplasmic vesicles. The release of mediators is thought to occur through small vesicle trafficking to the plasma membrane and secretion apparently independent of large granule events[116]. It is a process that can develop slowly over days. However, recent observations in our lab indicated that rapid mobilization of small secretory vesicles coincide with secretion of the chemokine, RANTES, within minutes following stimulation of human eosinophils with IFN-γ[117]. The secretion of RANTES did not coincide with the mobilization and release of contents of crystalloid granules particularly major basic protein (MBP), suggesting that small vesicles are involved in the rapid release of some, but not all preformed mediators[117]. PMD is implicated in histamine release in human basophils. Small amounts of histamine are required for homeostasis (e.g., for regulation of microvascular tone) and these cells function by releasing such substances continuously, in small aliquots rather than by explosive discharge[118-121]. Basophils migrated into involved tissues from blood vessels and over time developed piecemeal losses of their granule content. Assessment of skin lesions over 6 days revealed significant increase in basophils undergoing PMD[122, 123]. Other evidence of PMD was observed in peripheral blood and tissues from patients with Crohn's disease, an important and serious inflammatory bowel disease[119].

1.1.2.1.3 Compound exocytosis

Compound exocytosis is defined as granule-granule fusions within the cytoplasm followed by a single large exocytotic event resulting from granule membrane fusion with the plasma membrane. Although, this type of degranulation was not delineated by Dvorak as a separate type of degranulation, she described guinea pig anaphylaxis as following: "granules had fused with each other, and subsequently with plasma membrane, forming a degranulation sac, containing membrane-free granules that open to the cell exterior by a narrow pore[113]". According to intracellular Ca^{2+} concentration studied by a patch-clamp technique two distinct patterns of exocytosis were observed in human basophils. The first one is a rapid and vigorous fusion of granules with plasma membrane over 5 min that coincide with classical pattern exocytosis. Another mode is a slow mobilization of granules and granule-granule fusion continued for 7-10 min that coincide with compound exocytosis[112].

Recent studies on skin mast cell indicted that IgE-dependent stimulation following activation of tyrosine kinases, or non-immunologic stimulation followed activation of G-proteins, induced characteristic compound exocytosis resulting in the liberation of preformed mediators[55, 124].

1.1.2.1.4 Cytolytic degranulation

Cytolytic degranulation is a major pattern associated with deposition of granular content extracellulaly and eventually cytoplasmic content with subsequent loss of cell integrity[125] (Figure 1.5.D). This type of degranulation was first described in guinea pig basophils stimulated by skin challenge with feeding populations of the argasid tick, *Ornithodorus tartakovskyi*. The majority of basophils in 72 h lesions exhibited cytotoxic alterations culminating in complete disintegration[125]. It was suggested that such changes result from contact with tick-derived toxins or enzymes.

1.1.2.2 SNAREs and the SNARE complex formation

Over 10 years ago Rothman and colleagues proposed a universal "docking and fusion particle" theory to explain vesicle docking and fusion. The story of the SNAREs starts with the ATPase, NSF (N-ethylmaleimide-sensitive factor). NSF, a trimeric cytosolic protein with three external domains (an amino-terminal, D1 and D2)[126, 127], was originally identified and characterized as an essential component of Golgi-derived vesicular trafficking. Later, it was discovered that the amino-terminal of NSF specifically binds to SNAP (soluble NSF attachment proteins - α-, β-, γ-)[128]. α-, β-SNAPs are ubiquitously expressed, whereas γ - SNAP is the brain-specific isoform[129].

Both SNAP and NSF are cytosolic proteins and are essential for several transport steps. α -SNAP and NSF form a distinct multi-subunit 20S particle. A search for distinct organelles that are able to recruit SNAPs and NSF to these membranes receptor(s) revealed a complex of three proteins[130].

The first model was proposed in 1993 by *Rothman et al.* and later modified in 1995. The proteins identified had previously been reported to localize to either the vesicle or plasma membrane in neurons[131, 132]. The main focus of research over last decade has been on identificationof the several isoforms of SNAREs. It is now established that this family has a critical role in not only neurotransmitter release at neuronal synapses, but also several other pathways of intracellular membrane trafficking[133-136], including inflammatory cells[106, 137, 138].

The first SNARE complex was identified in the neuronal cells and was composed of a synaptic vesicle-associated membrane protein (VAMP, previously termed *synaptobrevin*) and two plasma membrane proteins (syntaxin and SNAP-25 (synaptosome associated protein of 25 kDa)). Both syntaxin and VAMP are anchored to the membrane by a carboxy-terminal transmembrane domain, whereas SNAP-25 is peripherally attached to the membrane by palmitoylation of four cysteine residues in the central region of the protein. According to their location and function, SNAREs can be divided into two groups: vesicular SNAREs (v-SNARE), located primarily on the granule or vesicle membrane and target SNAREs (t-SNARE) which are mainly associated with the target plasma membrane[130] (Figure 1.6)[139]. Isoforms of SNARE complex are also classified on the basis of whether they contribute a conserved arginine (R) or glutamine (Q) residue to the central core region of the complex. This classification replaces the older nomenclature of v-SNARE and t-SNARE, which categorized isoforms according their cellular localization. The crystal structure of the SNARE core complex shows that one coil of syntaxin and VAMP, and two coils of SNAP-25 intertwine to form a four-stranded coiled-coil structure (Figure 1.7).

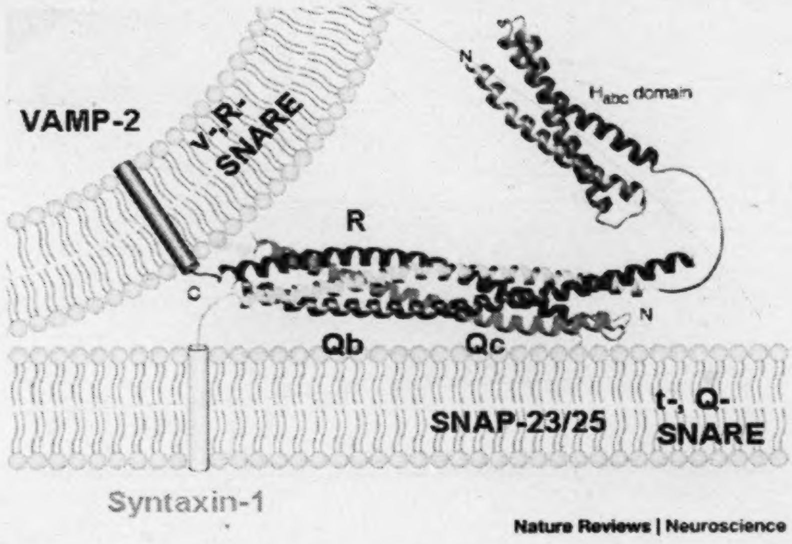
VAMP-2
v-, R-SNARE
H_{abc} domain
N
R
C
N
Qb
Qc
SNAP-23/25
t-, Q-SNARE
Syntaxin-1
Nature Reviews | Neuroscience

Figure 1-6 Model of the neuronal SNAREs assembled into the core complex.

The ribbon diagrams represent the crystal structure of the core complex and the NMR (nuclear magnetic resonance) structure of the amino-terminal H_{abc} domain of syntaxin-1. The H_{abc} domain is colored in orange and the SNARE (SNAP receptor) motifs are colour coded as follows: synaptobrevin, red; syntaxin-1, yellow; SNAP25 amino terminus, blue; SNAP25 carboxyl terminus, green. The cylinders represent the transmembrane regions of synaptobrevin and syntaxin 1, which are inserted into the synaptic vesicle and plasma membranes, respectively.

Modified from Rizo J.& Sudhof T Nature Rev. Neurosci. 2002 Aug;3(8):641-53.

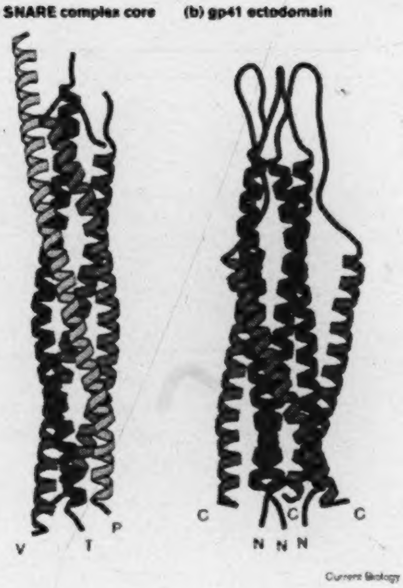

Figure 1-7 SNARE core structure.

The structure of the SNARE complex core. Four parallel αhelices from synaptobrevin (blue), syntaxin (red) and SNAP-25 (green) form the core of the synaptic complex. The carboxy-terminal transmembrane domains of synaptobrevin and syntaxin, a central 'linker' region of SNAP-25 and the amino-terminal regulatory domain of syntaxin are not present. Amino-terminal and carboxy-terminal segments of SNAP-25 (light and dark green, respectively) each contribute one helix separated by a 54-residue loop, a small part of which is visible just prior to the amino-terminal end of the carboxy-terminal helix (top). Membrane attachments are marked as V (vesicle membrane of synaptobrevin), T ('target' plasma membrane of syntaxin), and P (the beginning of the SNAP-25 linker)

Modified from Rizo J.& Sudhof T Nature Rev. Neurosci. 2002 Aug;3(8):641-53.

The structure of the SNARE core complex is an evolutionarily conserved hallmark of all SNARE complexes and is intimately associated with the general role of SNAREs in membrane fusion[140, 141]. The homology domain or "SNARE motif" that is common in all SNARE that constitute the superfamily consists of approximately 60 amino acids, forming a coiled-coil structure, which consists of repeating hydrophobic residues [142-144]. SNARE motifs assemble into parallel four helix bundles which are highly stable, being resistant to denaturation and unfold only when either heated to high temperatures or exposed to strong denaturants[145]. The core complex was found to be also resistant to cleavage by clostridial neurotoxins including tetanus (TeNT) and botulinum (BoNT) and to denaturation by SDS, highlighting its high stability. For such a stable complex, ATP is needed to dissociate it into monomeric components. Disassembly is carried out by two proteins, the ATPase NSF and an adaptor protein, α-SNAP (soluble NSF attachment protein), which were initially discovered as essential factors in Golgi transport. In this model, SNAPs and NSF are required to disassemble the core complex, recycling the SNAREs for another round of fusion (Figure 1.8).

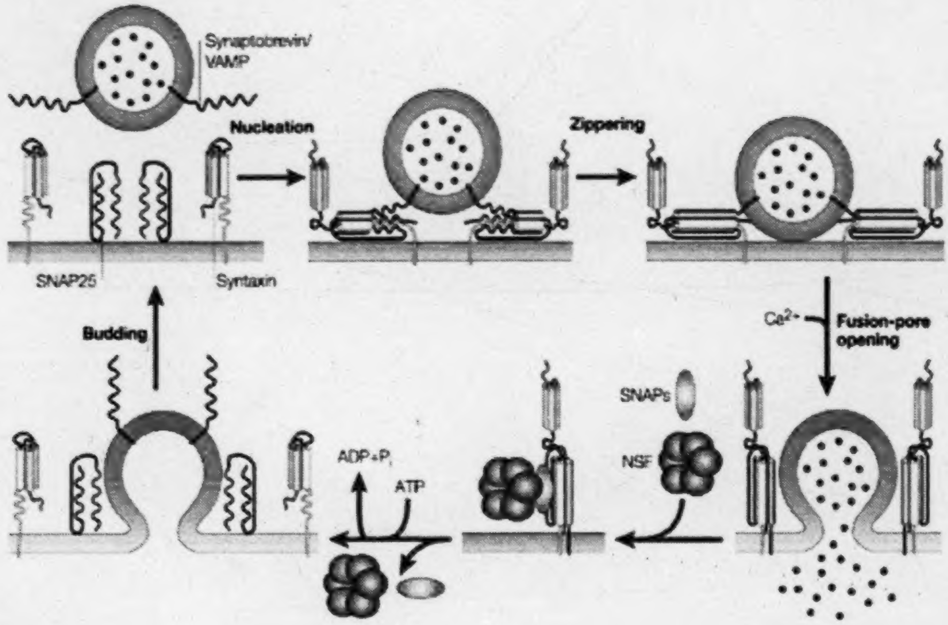
Synaptobrevin/
VAMP
Nucleation
Zippering
SNAP25
Syntaxin
Budding
Ca2+
Fusion-pore opening
SNAPs
NSF
ADP+Pi
ATP

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Figure 1-8 Cycle of assembly and disassembly of the SNARE complex in synaptic vesicle exocytosis.

Syntaxin exists in a closed conformation that needs to open to initiate core-complex assembly (nucleation). 'Zippering' of the four-helix bundle towards the carboxyl terminus brings the synaptic vesicle and plasma membranes towards each other, which might lead to membrane fusion. After fusion, *N*-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF-attachment proteins (SNAPs) disassemble the *cis*-core complexes that remain on the same membrane to recycle them for another round of fusion.

Modified from Rizo J.& Sudhof T Nature Rev. Neurosci. 2002 Aug;3(8):641-53.

SNARE isoforms are involved not only on the plasma membrane, but also in intacellular vesicular trafficking between ER and Golgi and endocytic transport in many eukaryotic cells, and their distribution varies in different cellular compartments (Figure 1.9). It has been proposed that formation of SNARE complexes between two membranes (*trans*-configuration) may be sufficient to overcome the energy barrier between the two opposing membranes to cause membrane fusion. The 'zipper' model of SNARE function postulates that the SNARE core complex 'zips' from the membrane-distal amino termini to the membrane-proximal carboxyl termini, and the formation of the stable SNARE complex overcomes the energy barrier to drive fusion of the lipid bilayers.

The functional importance of the synaptic SNAREs was first shown by the observation that they constitute the specific targets for clostridial neurotoxins, which inhibit neurotransmitter release[146, 147], and later by genetic experiments in *Drosophila.*

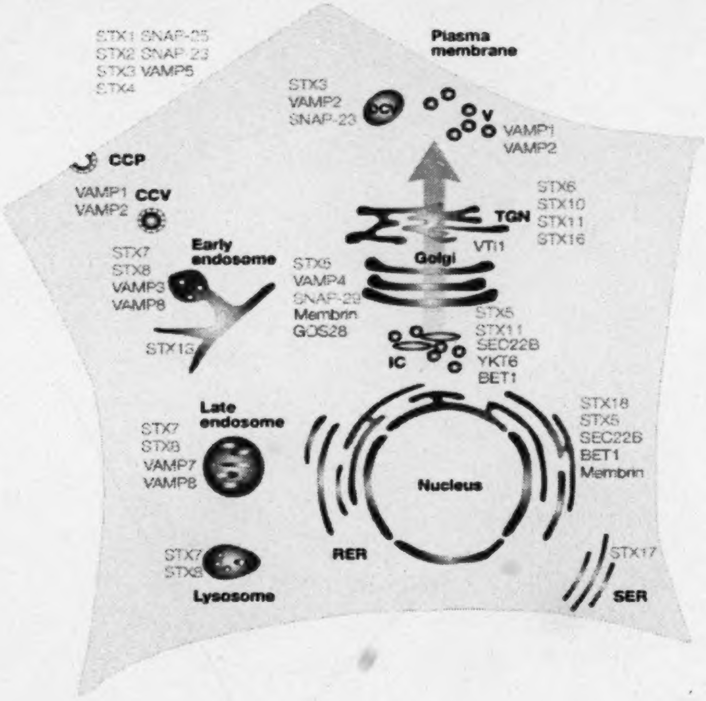
STX1 SNAP-25
STX2 SNAP-23
STX3 VAMP5
STX4
Plasma membrane
STX3
VAMP2
SNAP-23
DCV
V
VAMP1
VAMP2
CCP
VAMP1
VAMP2
CCV
STX6
STX10
STX11
STX16
TGN
VTi1
Golgi
Early endosome
STX7
STX8
VAMP3
VAMP8
STX13
STX5
VAMP4
SNAP-29
Membrin
GOS28
STX5
STX11
SEC22B
YKT6
BET1
IC
Late endosome
STX7
STX8
VAMP7
VAMP8
STX18
STX5
SEC22B
BET1
Membrin
Nucleus
RER
STX7
STX8
Lysosome
STX17
SER

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Figure 1-9 Subcellular localization of mammalian SNAREs.

The mammalian SNAREs that have been studied so far localize to distinct subcellular compartments in the secretory pathway. (Red, syntaxin family; blue, VAMP family; green, SNAP-25 family; black, others. CCP, clathrin-coated pits; CCV, clathrin-coated vesicles; DCV, dense core vesicles; IC, intermediate compartment; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; SNAP-25, 25 kDa synaptosome-associated protein; TGN, *trans*-Golgi network; V, vesicles; VAMP, vesicle-associated membrane protein.)

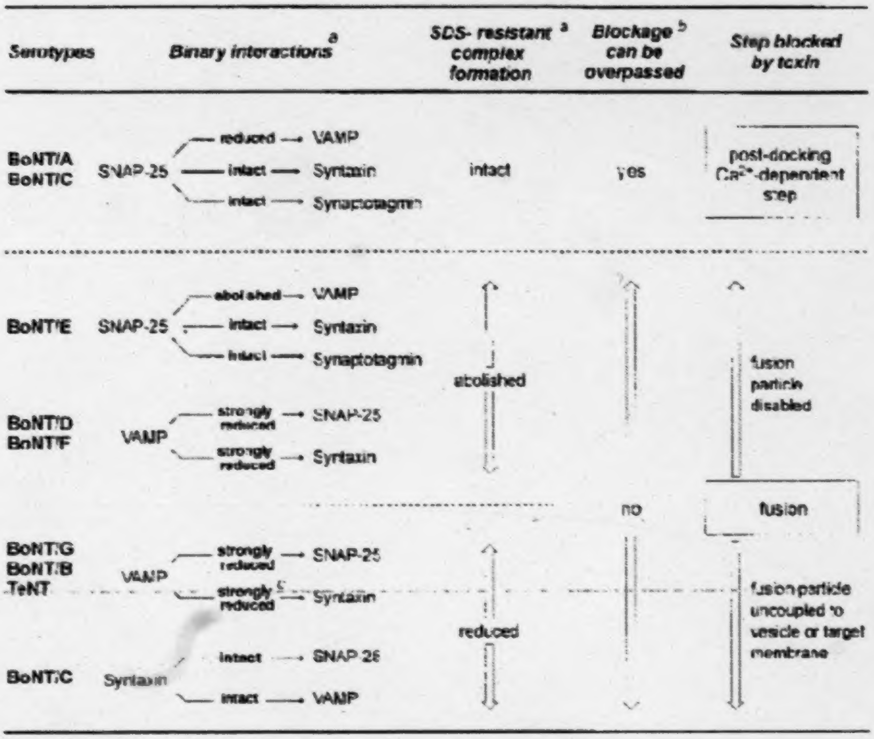
Adopted from: Chen YA, Scheller RH. Nature Reviews Molecular Cell Biology 2; 98-106 (2001)

1.1.2.3 Enzymatic cleavage of SNAREs: role of neurotoxins

1.1.2.3.1 Clostridial toxins

The clostridial neurotoxins (CNTs) have been among the most important tools in understanding exocytotic pathways. CNTs are the products of *Clostridium botulinum* and *C. tetani* and are synthesized as single polypeptide chains of ~150 kDa. Originally thought only to block neuronal function, selectivity of binding and uptake was eventually shown to reside with the heavy chains of these toxins, while the catalytic light chains (Zn^{2+}-dependent metalloproteinases) were the active components that effect blockade of neurotransmitter exocytosis[148, 149]. CNTs are well recognized for their ability to drastically impair secretory function in neurons and endocrine cells due to specific cleavage of SNARE isoforms[150, 151]. However, ternary SNARE complexes are resistant to CNT cleavage, perhaps due to masking of either the cleavage site and/or the toxin-binding regions[152]. Therefore, the ability of CNTs to impair membrane trafficking depends on the accessibility of SNARE-binding sites. Exocytotic steps blocked by clostridial neurotoxins are outlined in Table 1.3.

Table 1-3 Exocytotic steps blocked by clostridial neurotoxin.



Serotypes	Binary interactions[a]			SDS- resistant[a] complex formation	Blockage[b] can be overpassed	Step blocked by toxin
BoNT/A BoNT/C	SNAP-25	reduced	VAMP	intact	yes	post-docking Ca^{2+}-dependent step
		intact	Syntaxin			
		intact	Synaptotagmin			
BoNT/E	SNAP-25	abolished	VAMP	abolished		fusion particle disabled
		intact	Syntaxin			
		intact	Synaptotagmin			
BoNT/D BoNT/F	VAMP	strongly reduced	SNAP-25			
		strongly reduced	Syntaxin			
					no	fusion
BoNT/G BoNT/B TeNT	VAMP	strongly reduced	SNAP-25	reduced		fusion particle uncoupled to vesicle or target membrane
		strongly reduced[c]	Syntaxin			
BoNT/C	Syntaxin	intact	SNAP-25			
		intact	VAMP			

The molecular mechanisms used by clostridial neurotoxins to block exocytosis appear to fall into three categories (i) BoNT/A blocks a post-docking priming step, (ii) BoNT/D, /E, and /F prevent formation of fusion complex, (iii) BoNT/B, /C, /G, and TeNT uncouple fusion particle from vesicle or plasma membrane

Adapted from: Humeau Y. et al. 2000. Biochimie. 82(5) 427-446.

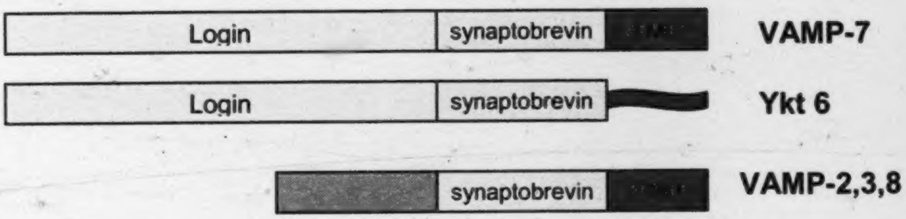
1.1.2.3.2 Botulinum and tetanus toxins

The identification and classification of SNARE proteins were paralleled with the discovery of their selective cleavage by BoNT and TeNT toxins[150,153,154]. Many VAMP/synaptobrevin isoforms are cleaved by TeNT and BoNT B, D, F and G; SNAP-25 isoforms by BoNT A, C and E; and syntaxins by BoNT C[148, 151, 155] .The selectivity of BoNTs and TeNT is mediated by their interaction with a specific binding region on the SNARE protein.

Assembly of SNARE complexes may be inhibited by different types of toxins. BoNT-D, E and F all impair SNARE complex formation. However, the cleavage product of VAMP-2 resulting from BoNT-B, G and TeNT treatment can still form complexes[156], but is dissociated from the vesicle membrane[157]. Numerous trafficking steps have been shown to be resistant to TeNT and BoNT treatment. A SNARE-independent mechanism[158] was postulated to be involved in observations of TeNT-resistant vesicle trafficking. However, this trafficking has been shown to be mediated by a tetanus-insensitive VAMP (VAMP-7) isoform[159-161]. Similarly, other SNARE-mediated trafficking steps may either be resistant or sensitive to these toxins, due to sequence variations within the CNT cleavage sites of the SNARE isoforms. These two categories of exocytic R-SNAREs define two important routes to and from the plasma membrane: one sensitive, the other insensitive to tetanus neurotoxin[160, 162].

The R-SNARE can be subdivided into short VAMPs or "brevins" (from the Latin word *brevis,* meaning short) and long VAMPs or "longins", on the basis of whether they contain a short and variable domain or a conserved long domain of 120-140 amino acids at their N-terminus[163]. Longins are found in yeasts, plants, animals and humans suggesting an important and evolutionary conserved function.

Recent studies by Galli have provided sufficient evidence that VAMP-7 is critically important in the maturatiom of axon terminals during brain development of rats[163-165]. It has previously been reported that VAMP-7 was localized in the somatodendritic compartment of neurons indicating a role in membrane fusion events within dendrites. There are a sufficient number of studies demonstrating that R-SNARE is also involved in vesicle release from non-neuronal tissues[166-168].



Longins domain: □
Transmembrane domain: ■
Specific N-terminal domain: ▨

Figure 1-10 Domain structures of longins and brevins.

In addition to the conserved synaptobrevin domain common to all VAMP species, tetanus-insensitive VAMP (VAMP-7) and related isoforms (eg: Ykt-like family members such as the *S. cerevisiae* R-SNARE, Ykt6p) contain an additional amino terminal region of approximately 110 residues termed the longin domain. Brevins (such as VAMP-2, -3 and -8) and the longin, VAMP-7 both have transmembrane domine (TMD) YKT-like longins have an isoprenyl anchor (zig-zag line) instead of a transmembrane domain. The green square in the structure of brevins represents a variable N-terminus and the black rectangles at the C-termini of brevins and longins represent intravesicular tails.

Modified from: Filippini F. et al. 2001. Trends. Biochem. Sci. 26(7) 407-409

1.2 STUDY OBJECTIVES AND HYPOTHESIS

The intracellular mechanisms and signaling molecules involved in the regulated exocytosis in human basophils are poorly understood. The identification of proteins involved in the process of degranulation/exocytosis are critical to the understanding of the increasing pro-inflammatory role of human mast cells and basophils. This may also provide novel therapeutic targets aimed at inhibiting the release of mediators from these cells. In accordance with the SNARE hypothesis, the expression and localization of distinct SNARE isoforms may regulate the secretion of different granule compartments. Previous studies indicated that there are some similarities in SNARE isoforms profile and mechanisms of granule/vesicule mediator release between neutrophils and eosinophils (M Logan, PhD thesis)

Almost all previous experimental work with identification and characterization of SNARE proteins was done on the RBL-2H3 mast cell line, a widely used model to study the biochemical events that follow the triggering of the FcεRI. However, in our study we used a newly established, *in-vitro*-derived mature human MC, the growth factor-dependent human MC line LAD-2[169] (a kind gift from Drs A.S. Kirshenbaum and D.D. Metcalfe, NIH, Bethesda, MD). There is no evidence in the literature whether or not LAD-2 cells express SNARE proteins.

Recent studies by *Zhenheng Guo et al* using SNARE-specific antibodies, showed that RBL express multiple syntaxin isoforms including syntaxins 2, 3 and 4 as well as SNAP-23[170]. VAMP proteins including VAMP-2, -3, VAMP-7 and -8 (endobrevin) were also expressed in RBL cells[171]. The fuctional involvement of SNARE proteins were determined by overexpression of certain proteins, such as syntaxin-4. It was shown that overexpression of syntaxin-4, but not syntaxin 2 or 3, inhibited the IgE-dependent degranulation response, thus providing evidence for a functional role for this SNARE heavy chain. Coimmunoprecipitation experiments in RBL cells revealed that syntaxin-4 not only interacts with SNAP-23, but also with several R-SNAREs isoforms including VAMP-2, -3 and –8[172]. Furthermore, introduction of anti-SNAP-23 antibodies into permeabilized rat peritoneal mast cells (RPMC) substantially inhibited the degranulation response[170]. To summarize knowledge of SNARE proteins *Blank* proposed the unifying hypothetical model of mast cell degranulation[173]: VAMP-2,-3,-7,-8/syntaxin-4/ SNAP-23. The role of R-SNARE proteins in degranulation may be complicated by the potential involvement of multiple partners. Unlike VAMP-2 and VAMP-3, both VAMP-7 and VAMP-8 isoforms are resistant to proteolytic cleavage by neurotoxins. Endogenous VAMP-2 localizes to punctuate granular structures in RBL cells, however its direct co-localization with a granular marker has not been demonstrated.

It is conceivable that distinct SNARE complexes could be implicated in mast cell degranulation. First, compound exocytosis in mast cells implicated the presence of several fusion processes including fusion between the granules themselves. As such, several R-SNAREs could be necessary for each of these processes. Additionally, mast cells are known for heterogeneity of their granules and therefore degranulation may involve different types of granules carrying potentially different R-SNARE molecules[174].

In contrast to mast cells, SNARE protein expression in human basophils has not been previously elucidated. Based on evidence from the literature of SNARE proteins involvement in exocytosis in other inflammatory cells, we hypothesized that:

Basophil secretion of stored mediators is regulated by isoforms of the SNARE complex.

The specific aims of this study are:

1. To define mRNA and protein expression of SNARE isoforms in human basophils and mast cells;

2. To localize granule (CD63$^+$) and vesicle stored products (IL-4) with SNARE proteins in human basophils and mast cells;

3. To investigate the functional role of selected SNARE isoforms in the release of cytokines by basophils by the introduction of SNARE-specific antibodies into permeabilized cells.

The results of these studies have an important implication for our understanding of basophil biology and development of novel therapeutic strategies to inhibit and/or block mediator release in allergic inflammation.

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2 Chapter

2.1 MATERIALS AND METHODS

2.1.1 Reagents

SNARE antibodies

Mouse monoclonal anti-human recombinant syntaxin-4 (IgG_1) was obtained from BD PharMingen (Mississauga, Ontario, Canada). Rabbit anti-SNAP-23 antiserum, affinity-purified rabbit anti-SNAP-23 and mouse monoclonal anti-VAMP-2 antibody (purified IgG1) were obtained from Synaptic Systems GmbH (Göttingen, Germany). Mouse mAb to human VAMP-7 clone 158.2 (directed against amino acids 119-188)[1] and affinity-purified anti-human VAMP-8 rabbit IgG (directed against the N-terminus peptide: MEEASGSAGNDRVRN)[2] were kind gifts from Dr T. Galli (INSERM, Paris, France) (Table 2.1). Mouse IgG1 isotype control was obtained from R&D Systems (Minneapolis, Minnesota). Rabbit serum, used as control antibody, was purchased from Jackson ImmunoResearch Laboratories Inc (West Grove, PA). Mouse IgG_1 (R&D Systems, Minneapolis, MN, USA); purified rabbit IgG_1 was obtained from Cedarlane Laboratories Ltd (Hornby, ON, Canada).

Table 2-1 Primary, secondary and isotype control antibodies.

Species	Name	Isotype	Company	Expected product size for WB	Western blot (WB)	Positive control for WB	Immunocyto chemistry	Flow cytometry	ELISA	Catalog No
Mouse	Syntaxin-4	Mouse IgG1	BD PharMingen	35kDa	+	Platelets	+		NA	S402220
	VAMP-2 (clone 69.1)	Mouse IgG1	Synaptic Systems GmbH	13kDa	+	Rat brain	+	NA	NA	104201
	VAMP-7 (clone 158.2)	Mouse IgG1	Gift from Dr. T. Galli	25kDa	+	Rat brain	+	+	NA	/
	IL-4	Mouse IgG1	eBioscience	15kDa	+	MC	+		+	14-709
	CD63	Mouse IgG1	BD PharMingen	53kDa			+	+	NA	556019
Rat	Syntaxin-6	IgG	BD PharMingen	30kDa	+	Rat brain	+	NA	NA	110062
Rabbit	SNAP-23 Polyclonal	IgG	Synaptic Systems GmbH	23kDa	+	Platelets	+		NA	111203
	VAMP-8	IgG	Gift from Dr. T. Galli	10kDa	+	Rat kidney	+	NA	NA	/

NA – not available

Other relevant antibodies

Rhodamine-Red-X-conjugated affinity purified donkey anti-rabbit IgG and Rhodamine-Red-X-conjugated affinity purified goat anti-mouse IgG were from Jackson Immunoresearch Laboratories Inc. (Westgrove, PA, USA). BODIPY-FL-labeled goat anti-mouse antibodies were purchased from Molecular Probes (Eugene, OR, USA). Phycoerythrin conjugated (PE) anti-rabbit and anti-mouse antibodies were purchased from Cedarlane laboratories (cat # CLCC43004). Mouse anti-human CD63 mAb (IgG_1) was purchased from BD Pharmingen. Streptolysin-O (SLO) (100 µg/ml stock) was purchased from Dr. S. Bhakdi (Johannes Gutenberg University, Mainz, Germany).

Other reagents

Phosphate buffered saline (PBS) : 0.14 M NaCl, 3.6 mM KCl, 2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$-H_2O, 0.5 mM $MgCl_2$-$6H_2O$, pH 7.2

Tris buffered saline (TBS) : 0.02 mM Tris base, 0.14 M NaCl, 3.8 ml 1N HCl in 1 L of H_2O

PCR Reagents : Superscript II reverse transcriptase and Taq Polymerase kits containing buffers, enzymes and dNTPs for RT and PCR were obtained from Invitrogen (Mississauga, ON).

Western blot cell lysis buffer : 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 µg/ml leupeptin

SDS loading buffer : 187.5 mM Tris-HCl, 6% SDS, 30 glycerol, 0.03% phenol red, 10% DTT (1.25)

10% acrylamide gel : 5 ml 1.5 M Tris-HCl, 10.3 ml ddH2O, 5 ml 40% acrylamide, 200 µl 10% SDS, 18 µl tetramethylethylenediamine (TEMED)

Transfer buffer : 50 mM Tris, 40 mM glycine, 20 % methanol.

Buffers for Human Basophil Purification :

HNS (Hanks' buffer): 0.9% normal saline + 10 mM HEPES, pH 7.35

HNS/BSA/EDTA : HNS + 0.5% BSA (2.5 g to 500 ml HNS) + 5mM EDTA (5 ml 0.5 M EDTA to 495 ml HNS), pH 7.35

Lysing buffer : 990 ml of H_20, 8.02 g NH_4Cl, 0.84 g $NaHCO_3$, 0.37 g EDTA

2.1.2 Isolation of human basophils from peripheral blood

Acid citrate dextrose (ACD), Ficoll-Histopaque®-1077 (Ficoll), PBS, EDTA, bovine serum albumin (BSA), *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphuric acid (HEPES); ammonium chloride lysing reagent (155 mM NH_4Cl, 10 mM $NaHCO_3$ and 0.1 mM EDTA) and modified Wright's stain were purchased from Sigma (St. Louis, MO); RPMI 1640 medium was obtained from Life Technologies (Burlington, ON); VarioMACS and VS Column with adapter were obtained from Miltenyi Biotec (Auburn, CA); StemSep™ Magnetic Colloid, biotinylated anti-human CD15 and StemSep™ Antibody Cocktail were purchased from StemCell Technologies (Vancouver, B.C.). For basophil isolation, this cocktail includes anti-biotin and bispecific tetrameric antibody complexes directed against dextran (on the iron-dextran magnetic particles) and cell surface markers including: glycophorin A, CD3, CD24, CD14, CD15 biotin, CD34, CD45RA, CD2, CD56, CD19, CD16, and CD36.

Venous blood (50 ml) was collected after informed consent according to the Helsinki protocol. Samples were obtained from subjects with CML, as well as atopic donors with peripheral basophil counts of $\geq$3%, without a history of recent steroid or cytotoxic medication. Basophils were highly enriched by negative selection using immunomagnetic beads (StemSep™ Antibody Cocktail) following initial separation by Ficoll density gradient centrifugation[3]. StemSep™ Magnetic Colloid, biotinylated anti-human CD15 and StemSep™ Antibody Cocktail was used. For basophil isolation, the cocktail included anti-biotin and bispecific tetrameric antibody complexes directed against dextran and cell surface markers. Isolated cells at a final purity of >95% were ≥98% viable as determined by trypan blue exclusion. The purity of the final basophil suspension was determined by staining with Wright/Giemsa.

Protocol for Basophil Isolation :

- Blood 50 ml was collected into syringe (10 ml of ACD in it)
- 60 ml of anticoagulated peripheral blood with ACD was centrifuged for 20 min at 190 g to remove the platelet-rich plasma
- Centrifuge at 400 g X 30 min, meanwhile prepare 3 tubes with 10 ml of Ficoll on ice. Vacuum the serum.
- Dilute the blood with Hanks' buffer (0.9% normal saline, 10 mM HEPES), add HNS to each tube to 37.5 ml, and divide into three tubes (25mL each)
- The 25 ml of diluted blood was overlaid onto Ficoll - Hypaque 1077 (10ml) and centrifuged at 500 g for 30 min at 4° C (each 25 ml of blood overlaid into three tubes with 10 ml of Ficoll 1077 in it)
- After centrifugation, the peripheral blood mononuclear cells fraction (PBMC) containing basophils, lymphocytes and monocytes was washed twice with lysis buffer (155 mM NH_4Cl, 10 mM $NaHCO_3$ and 0.1 mM EDTA) to lyse contaminated erythrocytes.

- This mononuclear fraction containing 1-3% basophils was incubated with 225 µl HNS/BSA/EDTA solution (0.5 BSA, 5 mM EDTA), 100 µl of StemSep™ antibody cocktail containing antibodies against the following (glycophorin A, CD2, CD3, CD24, CD14, CD15 biotin, CD34, CD45RA, CD56, CD19, CD16, and CD36) and 15 µl anti-CD15 in a tube rotator at 4° C for 15 min. Following further incubation for 15 min at 4° C with 60 µl of StemSep™ madnetic colloid (shake it before use) in total of 1 ml suspension volume, the unlabeled cells were passed through the magnetic column, while the labeled cells were retained on the magnetic column.

Purity of isolated basophils determined by Wright/Giemsa staining was greater then 95%. Human basophils were identify by multi-lobed nucleus (usually has a bilobed nucleus), uniformed large specific granules (with Wright/Giemsa staining cherry-purple in color) that cover the nucleus. Basophils are slightly smaller then neutrophils and nucleus is less segmented.

2.1.3 Cell lines

The human basophilic leukemia cell line, KU-812 (a kind gift from J.S. Marshall, Halifax, NS) was cultured in RPMI 1640 (Life Technologies, Burlington, ON) supplemented with 10% FBS, 2 mM glutamine, 10 mM HEPES and 40 U/mL penicillin/streptomycin. KU812 cells were treated with 0.3 mM sodium butyrate (3 d) before use to enhance granulation and maturation[4]. The cells were passaged twice per week. After culture, differentiated KU812 cells were washed twice and resuspended at the final concentration 1×10^6/ml in experimental medium before analysis. As a source of human mast cells, we used the growth factor-dependent human MC line LAD-2 (a kind gift from Drs A.S. Kirshenbaum and D.D. Metcalfe, NIH, Bethesda, MD). The human mastocytoma cell line, HMC-1, was kindly provided by J. H. Butterfield (Mayo Clinic, Rochester, MN)[5]. Cells were maintained in suspension culture at high density in Iscove's modified Dulbecco's medium supplemented with 10% FBS and 2 mM glutamine as described previously[6].

LAD-2 was cultured in StemPro-34 medium (Life Technologies) supplemented with 100 ηg/ml recombinant human stem cell factor (rhSCF) (Peprotech, Rocky Hill, NY) as previously described[7]. LAD-2 cells were passaged every 7-10 d. LAD-2 is a newly established mast cell line from a patient with the mast cell sarcoma–leukemia. Although LAD-2 and human mast cells-1 (HMC-1) appeared to share some similarities (such as histamine and tryptase release), only LAD-2 carry functional FcεRI and FcγRI (CD64) receptors[8]. Furthermore, HMC-1 derived from the peripheral blood cells, compare to LAD-2 that was established from bone marrow aspirate and is dependent on the cytokine stem cell factor (SCF). Moreover, *c-kit* is recognized as a receptor for SCF and point mutation in *c-kit* (codon 816) in HMC-1 causes substitution of valine for aspartate in the activation loop of the enzymatic pocket, leading to SCF-independent phosphorylation of the KIT protein. LAD-2 cells do not exhibit a *c-kit* mutation, which may explain the requirement for SCF for continued proliferation.

Rat basophilic leukemia (RBL-2H3) cells were cultured in Dulbecco's modified Eagle's Medium supplemented with 15% FBS. All cells were maintained at 37°C in a humidified incubator at 5% CO_2.

2.1.4 Primer design

Primer sets for the housekeeping gene GAPDH and SNAREs were designed and compared to known gene sequences in the GENBANK using websites of the National Center of Biotechnology Information (NCBI). Primer sequences for SNAREs were directed to genes coding for human VAMP-2 (Genbank Accession No. NM_005638), VAMP-7 (NM_005638), VAMP-8 (NM_003761), syntaxin-4 (AF026007), syntaxin-6 (NM_005819) and SNAP-23 (U55936). Primer sequences and their expected product sizes are listed in the Table 2.2.

Table 2-2 Primers Used in RT-PCR for Amplification of SNARE Proteins

SNARE isoforms	Size (bp)	Primers	
		Forward	Reverse
VAMP-2	348	ATGTCTGCTACCGCTGCCACG	AGAGCTGAAGTAAACTATGATGATG
VAMP-7(1)	684	AGACTGAAGCCATGGCGATT	CTATTTCTTCACACAGCTTGGC
VAMP-8(2)	587	GGCGAATTCACTTACTGACC	GTCTCTCCAGCCCACTCTAA
SNAP-23	498	CTGGGTTTAGCCATTGAGTCTCAGG	GGTGTCAGCCTTGTCTGTGATTCG
Syntaxin-4	894	ATGCGCGACAGGACCCACGAGCTG	TTATCCAACCACTGTGACGCCAATGAT
Syntaxin-6(3)	549	CCCTTCTTTGTGCTTCAGCA	CTGGGACATGTTCTTCAGCA

(1)-NIH Genbank Access. No. NM_005638

(2)-NIH Genbank Access. No. NM_003761

(3) -NIH Genbank Access. No. NM_005819

VAMP-2[9, 10], SNAP-23[11] and Syntaxin-4[9, 12] were designed in our laboratory from previously published reports. PCR products were cloned and sequenced to confirm specificity of products following a BLAST sequence similarity search over the website of the National Center for Biotechnology Information (NCBI) in our lab in previous studies on human eosinophils and neutrophils. All other primers were designed using the Primer3 primer design program.

(http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)

Amplified products were subcloned and sequenced in our lab in previous studies on human neutrophils and eosinophils. Briefly, PCR products were subcloned into the pCR™ 2.1 plasmid vector using the TA cloning kit (Invitrogen Corporation, Carlsbad, CA) and double-stranded plasmid DNA isolated with the Wizard Plus Mini-prep kit (Promega Corporation, Madison, WI) as previously described[13]. Plasmid DNA, containing the inserted PCR product, was sequenced using M13 forward and reverse primers and carried out on an ABI 373A automated sequencer (Applied Biosystems, Foster City, CA).

2.1.5 RNA extraction

Total RNA was extracted from basophils (1 x 10^6 cells minimum/extraction) with Trizol reagent (Invitrogen) according to the protocol of the supplier. This method routinely generates 0.2 to 1 µg RNA/10^6 cells. Cells were solubilized in Trizol (1 ml / 5 to 10 x 10^6 cells) and incubated for 5 min. A volume of 0.2 ml chloroform per 1 ml Trizol was added and tubes shaken vigourously for 15 s. Tubes were centrifuged at 12,000*g* for 15 min at 4°C. The aqueous supernatant was collected and transferred to a clean tube. To this was added 0.5 ml isopropanol/ 1 ml Trizol. The tubes were incubated for 10 min at room temperature and then centrifuged at 12,000 g for 10 min at 4°C. The RNA pellet was washed once in 75% ethanol and pelleted by centrifugation at 7,500 g for 5 min. Pellets were dried under vacuum for 30-60 min, resuspended in RNase-free water (10 µl/sample) and stored at -80°C until use. Absorbance values were measured in spectrophotometer at 260- 280 nm to determine the final RNA concentration.

2.1.6 Reverse-transcription-polymerase chain reaction (RT-PCR)

RT-PCR was carried out as follows: 1 μg total RNA was added to 1 μl oligo-(dT) (500 μg/ml) and diluted to 11 μl with RNase free water. The mixture was heated for 10 min at 70 ° C, following which 7.5 μl of RT master mix was added per reaction (4μl 5X First stand buffer (Gibco/BRL), 2μl 0.1 M DTT, 1 μl mixed dNTPs (10 mM), 1 μl RNase-free water). The reactions were incubated for 37 C for 1 h following the addition of 1 μl of M-MLV (200 units). Reactions were terminated by heating at 70°C for 10 min. cDNA generated was stored at –20°C or used immediately for PCR reactions.

PCR reactions were carried out in 20 μl volume according to the following protocol. 2 μl of cDNA was added to 17.5 μl of PCR master mix. 0.5 μl of Taq polymerase was added immediately before amplification. Reactions were carried out in a PTC 100 Thermal Controller (M-J Research, San Francisco, CA) according to the following program: Step 1: 5 min at 94 ° C, Step 2: 45 sec at 94 ° C, Step 3: 45 sec at 60 ° C, Step 4: 2 min at 72 ° C, Step 5: repeat Steps 2-4 for 30-40 cycles, Step 6: 7 min at 72 ° C, Step 7: 4 °C.

RT-PCR products were electrophoretically separated on a 2% agarose gel, stained with ethidium bromide (EtBr) and visualized by ultraviolet light. The product size was determined using a 2 Kb DNA molecular weight ladder (Sigma, Aldrich, ON).

As a positive control for Q- and R-SNAREs, we used cDNA from RBL cells[2, 14] while water was used as a negative reagent control. PCR products were cloned and sequenced to confirm specificity of products following a BLAST sequence similarity search over the website of the National Center for Biotechnology Information (NCBI) in our lab in previous studies on human eosinophils and neutrophils[13].

2.1.7 Western blot analysis

2.1.7.1 Protein isolation

Cells were resuspended in the cell lysis buffer and kept on ice for 30 min. Lysates of human platelets, rat brain and kidney were used as positive controls for SNARE protein expression (rat brain: VAMP-2, VAMP-7, syntaxin-6; rat kidneys: VAMP-8; platelets: SNAP-23 and syntaxin-4). Whole rat brain was homogenized, using a dounce homogenizer, in 3 ml of RIPA buffer containing 5 μg/ml protease inhibitor cocktail (5 μg/ml leupeptin, aprotinin and TAME, Sigma). Homogenate was incubated on ice for 30 min and nuclei/tissue debris removed by centrifugation at 7,000 g for 10 min. Supernatants were aliquoted and stored at –80 ^{0}C). For preparation of platelet lysates, peripheral blood (50 ml) was obtained from atopic donors, and subjected to centrifugation at 300 g for 10 min. Plasma-rich supernatant devoid of cells was harvested and centrifuged at 1800 g for 5-8 min. Platelet-rich pellet was resuspended in 1 ml 0.25 M sucrose, 10 mM HEPES, pH 7.4, and 1 mM EGTA containing 0.1% Triton X-100. The samples were centrifuged at 8,000 g for 10 min and stored at -80 C.

Before loading the samples, the supernatant from cells was diluted 1:3 with SDS loading buffer and boiled for 3 min.

2.1.7.2 Electrophoresis, transfer and detection of proteins

Samples and molecular weight markers were loaded on a 10% SDS polyacrylamide gel. Protein from electrophoresed gels were transferred to Immobilon PVDF membranes (Millipore, Bedford, MA, USA) using a semi-dry transfer (BioRad Laboratories) for 45 min at 15 V. Immobilon blots were incubated in 5% milk in TBS-T either overnight at 4 C or for 1 h. Primary antibodies were diluted in 5% milk in TBS-T with the exception of VAMP-7 antibodies (diluted in TBS-T alone, without milk) and incubated for 1 h at room temperature. Dilutions for antibodies were as follows: VAMP-2 mAb: 1:10000 (50 ng/ml); VAMP-7 mAb: 1:1000; VAMP-8 antiserum: 1:1000; syntaxin-4 mAb: 1:5000 (50 ng/ml); syntaxin-6 mAb: 1:1000 (250 ng/ml); SNAP-23 antiserum: 1:1000. Blots were washed 2X in TBS-T for 15 min. Secondary antibodies conjugated to horse-radish peroxidase, goat anti-mouse HRP and donkey anti-rabbit HRP (Amersham BioSciences, Baie d'urfe, PQ, Canada) were added (1:5000) for 1 h at room temperature. Blots were washed 4 X 5 min in TBS-T. Enhanced Chemiluminescence (ECL) substrate was added (Pierce SuperSignal, MJS Biolynx Inc., Brockville, ON, Canada) for 1 min to blots to drain excess substrate, and exposed to Hyperfilm (Amersham Biosciences) for 5-30 min exposure time. Band sizes were estimated by comparison of bands with the molecular weight standards using SigmaGel program (Jandel Scientific, San Raphael, CA).

2.1.8 Confocal laser scanning microscopy (CLSM)

Human basophils, KU-812 and LAD-2 cells were resuspended at 0.7-1 x 10^6 cells/ml in color-free RPMI 1640 containing 20% FBS and cytospins generated by centrifugation at 400 g (20,000 cells/slide) for 5 min. Slides were air dried for 1 h, wrapped in aluminum foil and stored at -20 ^{0} C until use. Slides were warmed to room temperature, re-hydrated for 1 min in PBS, then fixed in 2% paraformaldehyde (in PBS) for 5 min. Slides were washed in TBS, and blocked for 0.5 h in 5% goat serum in TBS.

Single labeling for t-SNAREs, CD63 and IL-4. Slides were incubated for 1 h with TBS containing 2.5 µg/ml affinity purified anti-SNAP-23 and 12.5 µg/ml anti-syntaxin-4. Purified rabbit IgG (2.5 µg/ml) was used as the negative control for anti-

SNAP-23, and mouse IgG_1 (12.5 µg/ml and 5 µg/ml) was used as the control for anti-syntaxin-4. SNAP-23 immunoreactivity was detected using 10 µg/ml Rhodamine-Red-X-conjugated affinity purified donkey anti-rabbit IgG. Syntaxin-4 immunoreactivity was detected using 14 µg/ml Rhodamine-Red-X-conjugated affinity purified goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc.). Images were captured using the Molecular Dynamics Confocal Imaging System 2001 (Molecular Dynamics, Sunnyvale, CA). Slides were examined using a 100× objective under a Leica CLSM (Heidelberg, Germany) with a x40 1.3 oil plan-Neofluar objective.

Double labeling of VAMP-2, 7 with CD 63 and IL-4. Slides were incubated for 1 h with 5 µg/ml anti-VAMP mAb or isotype control (mIgG1), washed 3X in TBS and labeled with 20 µg/ml BODIPY FL secondary antibodies. Slides were washed and blocked for 1 h in 10 µg/ml goat anti-mouse Fab fragment (Jackson Immunoresearch Inc.), following which they were incubated for 1 h with mouse anti-human CD-63 mAb (R&D Systems) at room temperature. Slides were washed in TBS and immunoreactivity was detected by incubation with 15 µg/ml goat anti-mouse IgG conjugated to Rhodamine Red (Jackson Immunoresearch Inc.)

Immunoreactivity to VAMP-2 and VAMP-7 was detected using 20 µg/ml BODIPY FL-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR) as previously optimized by our laboratory[15]. Mouse IgG1 (12.5 µg/ml) was used as the control for anti-VAMP-2 and anti-VAMP-7. Rabbit IgG (10 µg/ml) was used as the control for VAMP-8. To investigate co-localization of SNARE proteins with the granule marker, CD63, or the granule-stored cytokine, IL-4, a rabbit anti-CD63 or mouse anti-IL-4 monoclonal antibody were added to the respective slide and incubated in a dark room for 1 h in humid chambers. Immunoreactivity was detected with 10 μg/ml Rhodamine-Red-X–conjugated affinity-purified anti-rabbit IgG. Images were collected and processed as previously described[16]. Confocal analysis was carried out on labeled cells using the Molecular Dynamics Confocal Imaging System 2001 (Molecular Dynamics, Sunnyvale, CA) and Image Space 3.2 software for detection and imaging of immunofluorescence.

2.1.9 SLO permeabilization of KU-812 cells

Efficient transfer of antibodies into culture cells can be achieved by SLO-permeabilization of plasma membranes. SLO is a cytotoxic 69-kDa protein produced by strains of Streptococcus pyogenes and other β-hemolytic streptococci[17]. Membrane pores produced by SLO are large for passive diffusion of protein molecules. Previous studies demonstrated that the SNARE-specific antibodies can be successfully introduced into non-neuronal cell types[18].

Briefly, cells were washed 3 times in BSS (137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 20 mM PIPES, pH=6.8) and resuspended at 1 x 10^6 cells/ml. Cells were added in triplicate to v-well plates (50,000 cell/well) and permeabilized in 0.1-1.0 μg/ml SLO for increasing periods of time (0, 1, 2, 5, 10, 15 min). Exocytosis was induced by the addition of $Ca^{2+}$and GTPγS using Ca^{2+} EGTA-buffered BSS. Cells were incubated in either pCa 5, 5.5, 6.5, 7 supplemented with 10 μM GTPγS (activated) or pCa 7 in BSS alone (negative control) (note: pCa X= 10^{-x} M Ca^{2+}). Reactions were terminated at 10 min by the addition of 100 μl cold BSS, plates centrifuged at 350 g for 5 min at 4°C, and supernatants examined for β-hexosaminidase activity. Cell lysates (using 0.5% Triton-X-100) were used to determine total β-hexosaminidase activity. Values were expressed as either percent of total activity of β-hexosaminidase (detected in lysates) or % of maximal release (detected at pCa 5) according to the following formulae:

% total β-hex: $[(OD_{test} - OD_{neg\ control\ (pCa\ 7)}) / (OD_{lysed} - OD_{neg\ control\ (pCa\ 7)})] \times 100$

%maximal release: $[(OD_{test} - OD_{neg\ control\ (pCa\ 7)}) / (OD_{pCa5} - OD_{neg\ control\ (pCa\ 7)})] \times 100$

Results obtained from optimization experiments

The secretion of β-hexosaminidase from permeabilized KU-812 was elevated in response to increasing Ca^{2+} doses (Fig.2.1). A maximum secretion of 55% of total β-hexosaminidase activity was obtained at pCa 5 (n=3). Minimum effective of 0.1 μg/ml SLO was required to induce this response from KU-812 and was used for all subsequent experiments using SNARE antibodies. The doses of SLO used for these experiments are comparable to previously reported effective doses, which are known to vary according to cell type[19, 20], and were used for all subsequent secretion experiments[21, 22]. As a negative control we used pCa 7 that exhibited small spontaneous release values of 11% of β-hexosaminidase.

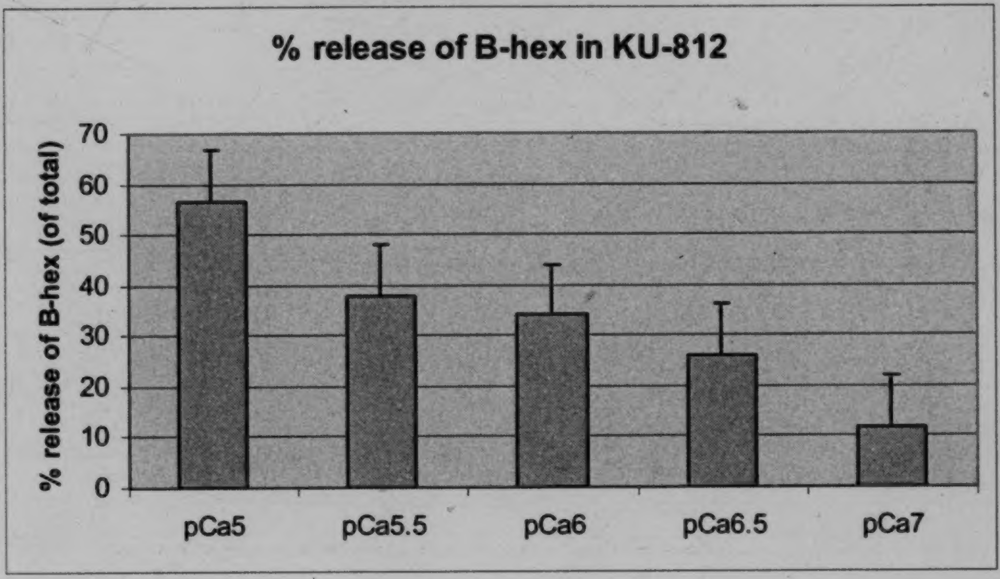

Figure 2-1 β-Hexosaminidase release (%) from permeabilized KU-812, following increasing concentration of Ca^{2+}.

KU-812 were permeabilized in 0.1 mg/ml SLO containing different doses of calcium (pCa7-pCa5) supplemented with 10 mM GTPγS. Negative permeabilized controls (pCa7) were incubated in the absence of GTPγS. Cell supernatants were examined for β-hexosaminidase release following 7 min incubation at 37° C, and % total release calculated as described in the Materials and Methods.

2.1.10 Analysis of mediator released from SLO-permeabilized cells.

Our study focused on two groups of storage compartments found in human basophils: the histamine-containing specific granules and small secretory vesicles. Upon stimulation human basophils and mast cells may release granular meditors such as histamine, heparin, chymase over 1-5 minutes. Small vesicles contain cytokines such as IL-4, IL-6, IL-8, IL-10, IL-13, and TNF and TGF-β. To determine the functional role of SNARE proteins in regulation of granule/vesicle exocytosis we developed two techniques to measure the selected mediator release.

2.1.10.1 β-Hexosaminidase assay

Because β-hexosaminidase is usually stored in the same compartment as histamine, β-hexosaminidase is an indirect way to measure the histamine release from cells. Therefore, it is generally accepted that β-hexosaminidase is degranulation marker for histamine storing cells.

KU-812 cells were pelleted at 200 g, and the supernatant was removed. Pellet and supernatant samples were assayed for ß-hex by hydrolysis of the fluorescent substrate 4-methylumbelliferyl *N*-acetyl-ß-D-glucosaminide (Sigma Chemical Co.; M-2133). Pellet and supernatant samples (50 µl) were added in duplicate wells in a microtitre plate followed by 1 mM substrate (50 µl), and the mixture was incubated at 37°C for 2 h as previously described[23]. The reaction was terminated by the addition of 100 µl 0.2 M Tris base, and the optical density (OD) was read at 450 nm (excitation 356 nm). The OD_{450nm} for blank wells, containing only substrate and Tris base, was automatically subtracted from the sample OD_{450nm}, and the percentage ß-hex release was calculated by the formula: OD_{450nm} of supernatant samples/(OD_{450nm} of pellet samples + OD_{450nm} of supernatant samples) x 100 = % release. The spontaneous release (in HTB) was subtracted from all samples to give % specific release.

2.1.10.2 Cytokine ELISA's assay (IL-4).

Cytokine ELISA assays were performed by Dr. John Gordon at Western College of Veterinary Medicine at the University of Saskatchewan. Immulon-4 flat-bottom plates (96-wells, Dynatech Laboratories Inc.) were coated with the appropriate capture antibodies in 50 µl volumes as previously described[24]. Rat anti-human IL-4 (PharMingen) was added at a concentration of 2 µg/ml. The plates were covered and incubated at 4°C overnight. The next day, the wells were washed twice with PBST and blocked with 200 µl of DMEM-10% FBS for 2 h at room temperature. Following another washing step, standards and samples were added to the wells in 100 µl volumes. Recombinant (r) IL-4 was diluted in Dulbeco's Minimum Essential Medium (DMEM) containing 10% Fetal Bovine Serum (DMEM-10% FBS), with standard curves ranging from 4000 pg/ml to 0.98 pg/ml for IL-4. Samples were plated undiluted in 100 µl volumes. Blank wells received 100 µl of DMEM-10% FBS instead of cell supernatants. The plates were covered and incubated at 4°C overnight. Biotinylated antibody (100 µl volume) was added the next day following four washes of the plates with PBST. Biotinylated rat anti-human IL-4 was added at concentration 1.25 µg/ml. The plates were incubated at 37°C for 1.5 h. Following six washes with PBST, 100 µl of streptavidin-conjugated horseradish peroxidase (Vector Laboratories Inc.), diluted 1:667 in PBS-10% FBS, was added to each well and the plates were again incubated at 37°C for 1 h. All samples were plated in triplicate. The level of sensitivity for IL-4 was 3.9 pg/ml. Results are expressed in pg/ml with reference to the standard curves.

Values from separate experiments (n=10) were pooled. Due to the variability among successive experiments, all data were standardized relative to the control values. Cells alone were defined as 100% release. We used the ANOVA test to determine the significance between examined conditions.

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3 CHAPTER

This chapter stands alone with the exception of Materials and Methods (see Chapter 2) as a separate document and is written in the style of the "Journal of Allergy and Clinical Immunology"

3.1 Expression of SNARE Proteins in Human Basophils and Mast Cell Lines: Potential Regulators of Exocytosis.

3.2 Abstract

Background: Mast cells and basophils synthesize and secrete a variety of proinflammatory mediators. Exocytosis of vasoactive amines, proteases, cytokines and chemokines is thought to contribute to tissue injury and remodeling. Different isoforms of SNARE proteins (soluble NSF attachment protein receptors) have been implicated in regulating vesicle and granule docking during exocytosis. However, little is known about membrane docking and fusion in basophils. **Objective:** We investigated expression of SNARE proteins in human peripheral basophils and a basophilic cell-line, KU-812. We compared these findings with those from two mast cell lines; human mast cell-1 (HMC-1) and the LAD-2 (Laboratory of Allergic Diseases-2). **Methods:** Human peripheral blood basophils (≥ 95% purity) from atopic and CML subjects were enriched by negative immunomagnetic selection, total RNA extracted and subjected to RT-PCR. Protein expression was determined by Western blot analysis and confocal microscopy. KU-812 were permeabilized with SLO, stimulated with Ca^{2+} and GTPγS and supernatants examined for surface CD63 (granular marker) expression and mediator release. **Results:** Human basophils (n = 7 donors) expressed mRNA for R-SNARE isoforms: vesicle-associated membrane proteins (VAMP) -1, -2, -3, -7 and –8, and the Q-SNAREs; SNAP-23 and syntaxin-4 and -6. KU-812, HMC-1 and LAD-2 expressed a similar SNARE profile to that in human basophils. Syntaxin-4, -6 and SNAP-23 and VAMP-7, VAMP-8 protein bands were also identified in basophils by Western blot analysis. Confocal analysis showed substantial co-localization of the R-SNAREs, VAMP-2, -8 and VAMP-7, with CD63, and with IL-4 in human basophils. In permeabilized cells VAMP-7, but not VAMP-8, antibodies impaired the secretion of IL-4. **Conclusions:** Our findings

suggest that human basophils express SNARE isoforms necessary for docking of their granules and secretory vesicles and share common exocytotic mechanisms with other inflammatory cells. Our observations indicate a novel role of VAMP-7 in human basophils.

3.3 Introduction

Mast cells (MC) and basophils are critically important cell types in allergic and inflammatory reactions. They synthesize and secrete a wide variety of vasoactive substances, proteases and cytokines, that contribute to tissue injury and remodeling[1]. Both human MC and basophils express the high affinity receptor for IgE (FcεRI) and synthesize and store histamine. It is likely, therefore, that they share similar intracellular mechanisms leading to exocytosis[2]. Basophils contribute to the immediate anaphylactic reaction through the release of histamine to the systemic circulation[3]. In addition, basophils are an important source of IL-4 and IL-13[4, 5], key cytokines in the pathogenesis of allergic inflammation. An intriguing feature of basophils is their ability to extravasate to sites of inflammation during the late-phase reaction. Thus, basophils play a key role in allergic disorders not only by secreting a plethora of inflammatory mediators, but also by promoting IgE synthesis and Th2 type immune responses. Despite their similar mediator profile, tissue MC and circulating basophils are distinct morphologically, ultrastructurally, immunologically and biochemically. Whether intracellular pathways of granule and vesicular transport, docking and exocytosis are similar between the two cell types remains unclear[6-8].

Granule and vesicle docking in association with exocytosis is critically dependent on SNAP receptors (SNARE)[9, 10]. Based on their location and function, SNARE proteins can be divided into two groups: vesicular SNARE (R-SNARE) molecules, located primarily on the granule or vesicle membrane, and target SNARE (Q-SNARE) proteins which are mainly associated with the target plasma membrane[11]. Exocytosis occurs via a series of exquisitely regulated sequential events, which include vesicle/granule tethering, docking, and membrane fusion with plasma membrane. It is hypothesized that particular sets of type-cell specific SNARE isoforms may regulate specific membrane traffic steps. v-SNARE proteins present on vesicles are hypothesized to recognize and form complexes with their cognate receptors, t-SNAREs, on other vesicles[12]. SNARE proteins are important in facilitating and promoting granule and vesicular docking in various cell types, including neuronal[13], endocrine[14] and inflammatory cells[15-17]. Isoforms of SNARE complex are also classified on the basis of whether they contribute a conserved arginine (R) or glutamine (Q) residue to the central core region of the complex. Recent experiments from our laboratory demonstrated that human eosinophils express the Q-SNAREs, SNAP-23 and syntaxin-4, which are predominantly localized to the plasma membrane, and the R-SNAREs, VAMP-2, VAMP-7 and VAMP-8[18, 19]. Recent studies on SNARE involvement in exocytosis of human neutrophils suggested a role for VAMP-2/syntaxin-4/SNAP-23 as well as VAMP-2/syntaxin-6/SNAP-23[16, 20-22]. By contrast, MC degranulation has implicated VAMP-2, -3, -7, -8/syntaxin-4/ SNAP-23[15, 23, 24].

In the absence of previous information on the intracellular mechanisms regulating exocytosis in human basophils, we studied SNARE expression and compared human basophils with basophilic and mast cell lines. We report for the first time on human basophil expression of various plasma and granule membrane SNARE proteins, with the potential to regulate exocytosis.

3.4 Results

3.4.1 Human basophil purification

One of our first objectives was to obtain a sufficient number of purified human basophils. To achieve this, we developed new sophisticated methods of isolation and purification of large number of peripheral blood basophils through collaboration with Dr. Robert R. Schellenberg (St Paul's Hospital, University of British Columbia) using negative selection with appropriately coated micromagnetic beads - CD2, CD3, CD7, CD14, CD15, CD16, CD19, CD24, CD36, CD45RA). Highly purified (>95%) human basophils from atopic and CML patients were obtained by negative immunomagnetic selection. The identity of these cells was confirmed by cytospin preparation and staining with Wright/Giemsa (Fig.3.1). Human basophils were identified by multi-lobed nucleus (usually as a bilobed nucleus), uniformly large granules (with Wright/Giemsa staining cherry-purple in color) that cover the nucleus. We were unable to detect structural and morphological differences among basophils from atopic, non atopic and CML donors. The main difference was the number of isolated basophils. On average, we were able to isolate 0.5-0.7x10^6/ml cells from atopic patients (50 ml of peripheral blood). In contrast, isolation from basophilic leukemic patients yielded over 5-7x10^6/ml basophils from the same amount of peripheral blood.

The human leukemia cell line, KU-812, had been described as an immature prebasophilic cell line[25]. It was derived from a patient suffering from CML, and has a number of characteristics of immature basophils. Although KU-812 expresses high-affinity FcεRI[25] and possesses high homology of surface receptor expression with mature human basophils, there are fundamental differences in terms of morphology and granularity, including lack of large metachromatic granules, non-segmented oval nucleus, and relatively larger size of the cells. Most of the functional involvement of SNARE proteins was done on KU-812 cells.

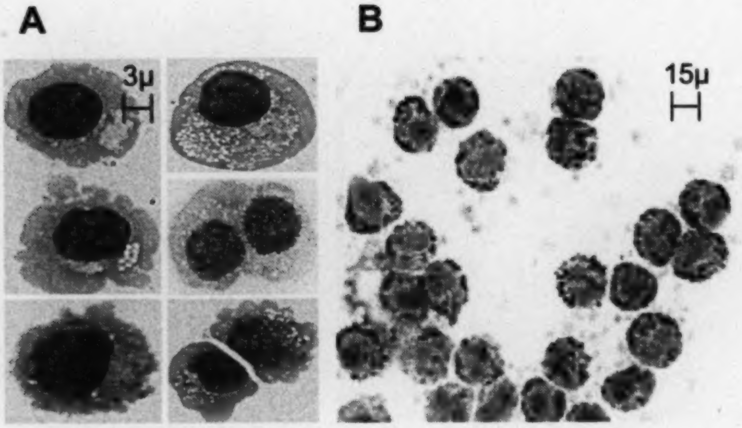

Figure 3-1 Morphology of human basophils and KU-812.

A. The human leukemia cell line, KU-812, had been described as an immature prebasophilic cell line. KU-812 cells have single nucleus and a few cytoplasmic granules with developed vacuoles.

B. Human basophils were identified by multi-lobed large nucleus (usually a bilobed nucleus), uniformly large granules (with Wright/Giemsa staining cherry-purple in color) that cover the nucleus. Cytospin preparation with Wright/Giemsa staining.

3.4.2 RT-PCR analysis for SNARE mRNA expression in human basophils, KU-812 and LAD-2 cells.

RT-PCR was used to identify the presence of transcripts encoding Q- and R-SNARE. Purified human basophils expressed mRNA for the R-SNAREs, VAMP-1, -2, -3, VAMP-7 and -8 (Fig. 3.2) and the Q-SNAREs, syntaxin-4, -6 and SNAP-23 (Fig. 3.3). Total RNA was obtained from different donors, reverse transcribed and amplified (30 cycles) prior to gel analysis. Lane 1 represents the DNA ladder. Lane 2 and 3 represent PCR products obtained using SNARE-specific primers for two different donors. Single PCR products at the predicted size co-migrated with appropriate positive control RBL cells as shown in lane 4 and 5. Lane 6 represents a housekeeping gene GAPDH (200 bp). Basophils from atopic, non-atopic and CML patients also expressed mRNA for all SNARE isoforms investigated (data not shown), suggesting that gene expression was not exclusive to allergic or myeloproliferative disorders. Sequences of the amplified fragments revealed >98% identity with the reported human cDNA sequences for the respective isoforms (sequencing was preformed in our lab during previous studies on eosinophils)[26].

We repeated the same set of tests in mast cells to identify the potential differences/similarities in expression of SNARE isoforms. PCR analysis revealed that LAD-2 expressed mRNA for the R-SNARE, VAMP-1, -2, -3, VAMP-7 and -8 (Fig. 3.4) and the Q-SNARE, syntaxin-4, -6 and SNAP-23 (Fig. 3.5). LAD-2 expressed identical SNARE isoforms to those in human basophils.

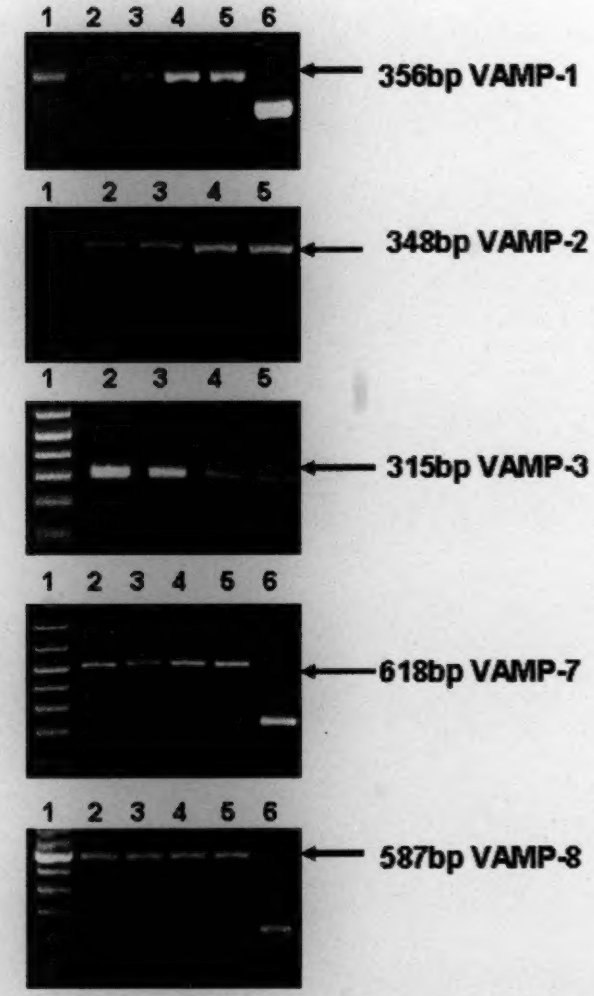
1 2 3 4 5 6
356bp VAMP-1
1 2 3 4 5
348bp VAMP-2
1 2 3 4 5
315bp VAMP-3
1 2 3 4 5 6
618bp VAMP-7
1 2 3 4 5 6
587bp VAMP-8

Figure 3-2 mRNA expression for R-SNARE isoforms in human basophils. Using specific primers for R-SNARE molecules, message encoding VAMP-1, -2, -3, VAMP-7 and VAMP-8, was detected by RT-PCR. Total RNA was obtained from different donors, reverse transcribed and amplified (30 cycles) prior to gel analysis. Lane 1 is a DNA ladder. Lanes 2 and 3 represent two samples from two donors of amplified product from basophil cDNA. Lanes 4 and 5 represent positive control for PCR products, using cDNA of RBL cells. Lane 6 represents a housekeeping gene GAPDH (200bp). The PCR products were electrophoresed and visualised with ethidium bromide. Primer sequences and conditions for cDNA amplification are detailed in Materials and Methods.

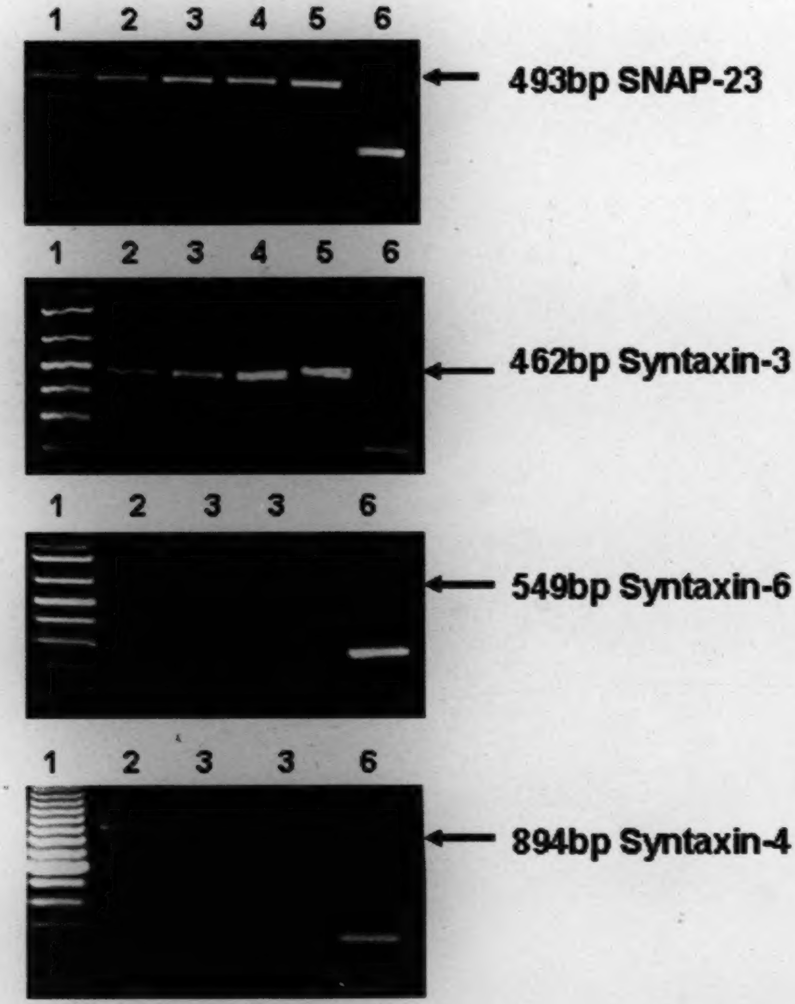
1 2 3 4 5 6
493bp SNAP-23
1 2 3 4 5 6
462bp Syntaxin-3
1 2 3 3 6
549bp Syntaxin-6
1 2 3 3 6
894bp Syntaxin-4

Figure 3-3 mRNA expression for Q-SNARE isoforms in human basophils. Using specific primers for Q-SNARE molecules, mRNA encoding SNAP-23, syntaxin-3, syntaxin-4 and syntaxin-6 was detected by RT-PCR. Lane 1 is a DNA ladder marker. Lanes 2 and 3 represent two samples from two donors of amplified product from basophils cDNA. Lanes 4 and 5 represent positive control for PCR products, using RBL RNA. Lane 6 represents a housekeeping gene GAPDH (200bp). The PCR products were electrophoresed and visualised with ethidium bromide.

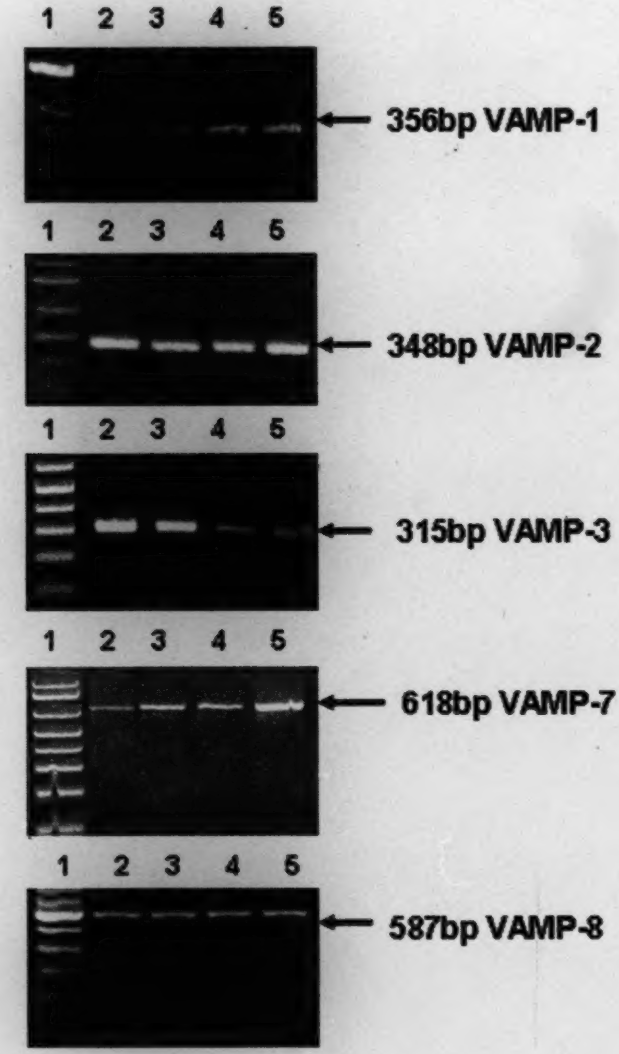
1 2 3 4 5
356bp VAMP-1
1 2 3 4 5
348bp VAMP-2
1 2 3 4 5
315bp VAMP-3
1 2 3 4 5
618bp VAMP-7
1 2 3 4 5
587bp VAMP-8

Figure 3-4 mRNA expression for R-SNARE isoforms in LAD-2 mast cells.

Using specific primers for R-SNARE molecules, mRNA encoding VAMP-1, -2, -3, VAMP-7 and VAMP-8 was detected by RT-PCR. Lane 1 represents the DNA ladder. Lanes 2 and 3 represent two samples of amplified product from LAD-2 mast cells cDNA. Lanes 4 and 5 represent positive control from RBL RNA. The PCR products were electrophoresed and visualised with ethidium bromide.

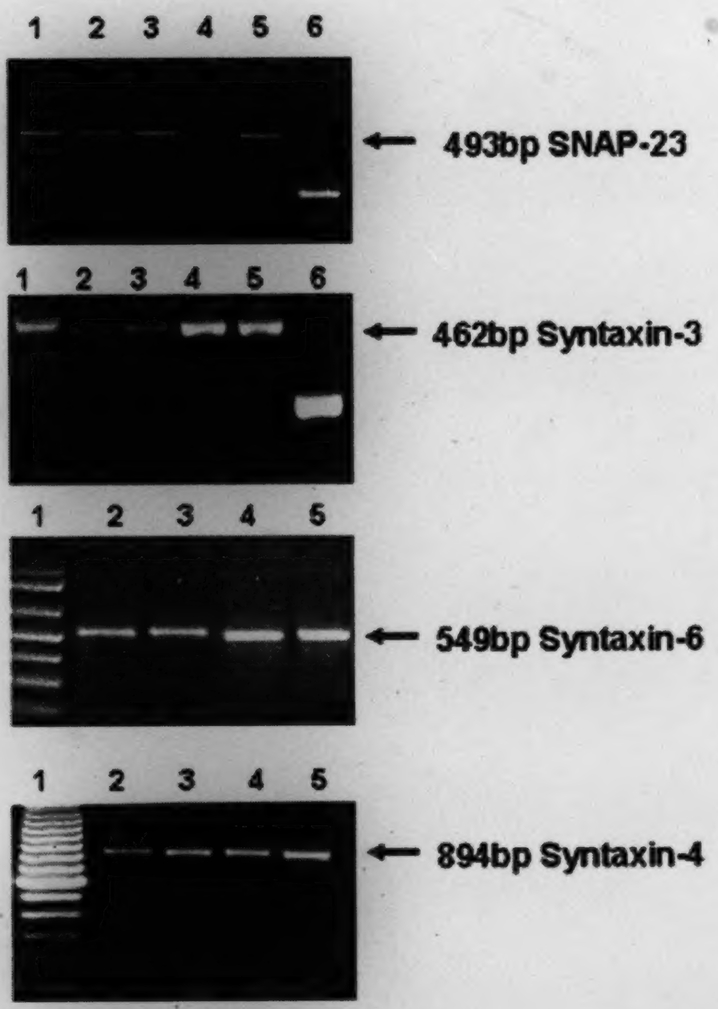
1 2 3 4 5 6
493bp SNAP-23
1 2 3 4 5 6
462bp Syntaxin-3
1 2 3 4 5
549bp Syntaxin-6
1 2 3 4 5
894bp Syntaxin-4

Figure 3-5 mRNA expression for Q-SNARE isoforms in LAD-2 mast cells.

RT-PCR detection of mRNA encoding for SNAP-23, syntaxin-3, syntaxin-4 and syntaxin-6. Lane 1 is a DNA ladder. Lanes 2 and 3 represent two samples of amplified product from LAD-2 mast cells cDNA. Lanes 4 and 5 represent positive control for PCR products, using cDNA of RBL cells. Lane 6 represents a housekeeping gene GAPDH (200bp). The PCR products were electrophoresed and visualised with ethidium bromide.

3.4.3 SNARE proteins expression in basophils, KU-812 and LAD-2 cells

To further characterize Q- and R-SNARE expression, we examined protein expression by Western blot analysis of whole cell lysates of human basophils, KU-812 and in both mast cell lines HMC-1 and LAD-2. Examination of Q-SNAREs in basophils demonstrated that syntaxin-4, -6 and SNAP-23 co-migrated to the expected molecular size comparable with appropriate controls (syntaxin-4 35 kDa, syntaxin-6 30 kDa and SNAP-23 23 kDa). (Fig. 3.6). We used rat brain as a positive control for syntaxin-6 and human platelets for SNAP-23 and syntaxin-4. Human basophils and KU-812 exhibited immunoreactivity for R-SNAREs, VAMP-7 and VAMP-8. All SNARE isoforms migrated similarly to that of their respective controls (VAMP-7 25 kDa, VAMP-8 10 kDa). Thus, isoforms of various protein components of the SNARE complex are expressed in human basophils. Interestingly, Western blot analysis for VAMP-7 consistently showed two bands (20 kDa and 25 kDa). The positive control co-migrated with the 25 kDa band. Previously, spliced variants of VAMP-1 were identified in primary human endothelial cells (VAMP-1A and VAMP-1B) [27]. However, it remains to be determined if the protein corresponding to 20 kDa is a potential alternatively spliced form of VAMP-7.

Similarly, we examined immunoreactivity of whole cell lysates from HMC-1 and LAD-2 cells. Both cell lines showed immunoreactivity for Q-SNARE proteins, SNAP-23, syntaxin-4 and -6 which co-migrated with their corresponding positive controls (apparent molecular weights of approximately 23 kDa, 35 kDa and 30 kDa, respectively). Mast cell lines exhibited immunoreactivity for R-SNAREs, VAMP-7 and VAMP-8. All SNARE isoforms migrated similarly to that of their respective controls. (Figure 3.7) In contrast to VAMP-7 in human basophils and KU-812, immunoreactivity to VAMP-7 in LAD-2 and HMC-1 with the appropriate size of the protein was less pronounced. Furthermore, the low band (20 kDa) was abundantly expressed. We did not detect the presence of VAMP-2 in cell lysates in neither human basophils nor the MC line, LAD-2 using Western blot analysis. Appropriate positive controls (rat brain) with VAMP-2 consistently corresponded to the expected product size.

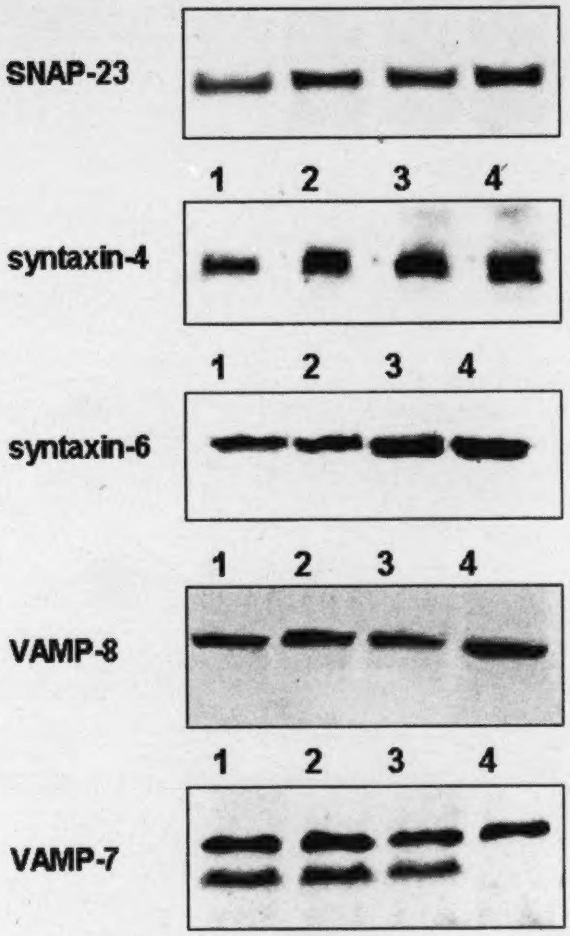
SNAP-23
1 2 3 4
syntaxin-4
1 2 3 4
syntaxin-6
1 2 3 4
VAMP-8
1 2 3 4
VAMP-7

Figure 3-6 Detection of SNARE proteins in human basophils and KU-812.

Lane 1 represents human basophil whole cell lysate (15 µg), lanes 2 and 3 – KU-812 (15µg), lane 4 is a positive control: rat brain (1 µg) for VAMP-7 and syntaxin-6, human platelets (5 µg) for SNAP-23 and syntaxin-4, rat kidney (5 µg) for VAMP-8. All SNARE isoforms migrated similarly to that of their respective controls (VAMP-7, 25kDa, VAMP-8, 10KDa, syntaxin-4, 35KDa, syntaxin-6, 30KDa and SNAP-23, 23KDa).

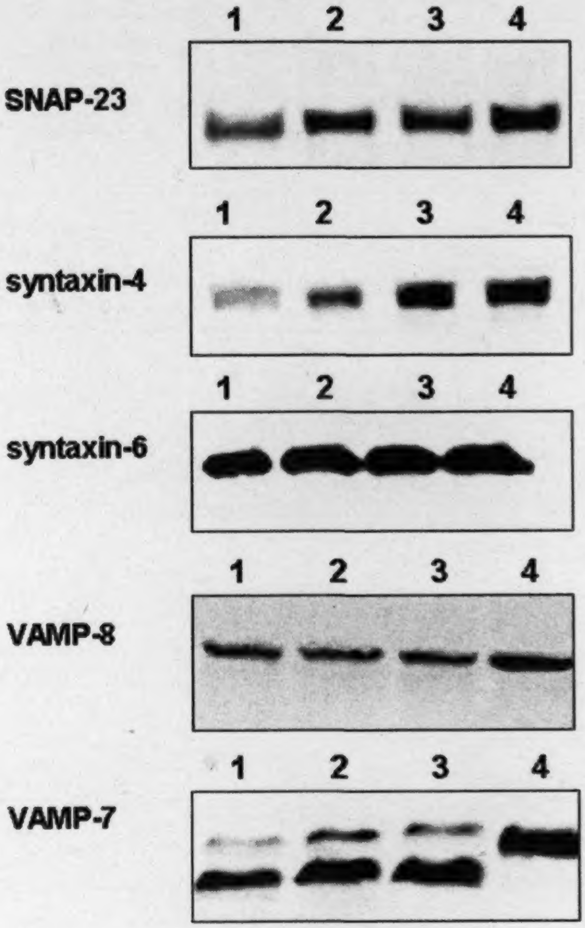
1 2 3 4
SNAP-23
1 2 3 4
syntaxin-4
1 2 3 4
syntaxin-6
1 2 3 4
VAMP-8
1 2 3 4
VAMP-7

Figure 3-7 Detection of SNARE proteins in the MC lines LAD-2 and HMC-1.

Lane 1 represents whole cell lysate for HMC-1, lanes 2 and 3 - LAD-2. Lane 4 is a positive control: rat brain (1 µg) for VAMP-7 and syntaxin-6, human platelets (5 µg) for SNAP-23 and syntaxin-4, rat kidney (5 µg) for VAMP-8. All SNARE isoforms migrated similarly to that of their respective controls (VAMP-7, 25kDa, VAMP-8, 10KDa, syntaxin-4, 35KDa, syntaxin-6, 30KDa and SNAP-23, 23KDa).

3.4.4 Co-localization of granule/vesicle products with SNARE isoforms by confocal laser scanning microscopy

3.4.4.1 Double staining of CD63 and SNARE-specific antibodies

We examined intracellular staining in human basophils, prebasophilic cell line KU-812 and mast cell line LAD-2 with specific R-SNARE antibodies and with the granule marker, CD63. Representative images of those cells were stained with BODIPY-FL-conjugated secondary antibody (green) to detect immunoreactivity against CD63 and Rhodamine-Red-X-conjugated secondary antibody (red) to detect VAMP-2 immunoreactivity (Fig.3.8). CD63 was scattered within the cytoplasm consistent with the distribution pg granules (lane 2). Similarly, VAMP-2 immunoreactivity (lane 3) showed coarse cytoplasmic distribution in resting human basophils. DIC (Differential Interference Contrast) was depicted in lane 4 for evaluation of cell integrity. It appears that a significant portion of cytoplasm expressed CD63 and VAMP-2 immunoreactivity in human basophils, which may be associated with cytoplasmic granules. Coinciding images of VAMP-2 (red) and CD63 (green) merged to generate the yellow fluorescence (lane 5). Immunofluorescence images demonstrated substantial co-localization of VAMP-2 and $CD63^+$ granules. We acquired images of negative controls in which the specific primary antibody was replaced by an irrelevant mouse IgG_1. An irrelevant mouse IgG1 replaced first primary antibody (CD63) or first secondary antibody (VAMP-2) was replaced with IgG_1 from processing. Isotypes control images were taken under the same settings as experimental images and showed no immunoreactivity to any of investigated antibodies.

Similar immunoreactivity was obtained in KU-812 cell and LAD-2 cell lines (Fig.3.9 A and B). We examined over 80 slides of KU-812 for immunoreactivity. We observed two distinct patterns of localization of VAMP-2 and/or CD63. One mode of immunoreactivity was uniform throughout the cytoplasm. In contrast, some KU-812 cells showed a unique perinuclear stain (yellow arrows). Previously, we have shown (Fig.3.1) that KU-812 lacks many metachromatically stained granules but has numerous vacuoles. The possibility cannot be excluded that CD63 maybe expressed in all those intracellular compartments. LAD-2 appeared to have a uniform distribution of CD63 and VAMP-2 throughout the cytoplasm. Our data on CD63 and VAMP-2 in human basophils coincided with previously reported results of intracellular co-localization of R-SNARE and CD63 in eosinophils[26]. In isotype controls, specific primary antibody was replaced by an irrelevant mouse IgG_1. No significant staining was observed when the secondary antibody was omitted from the processing. Isotype control images were taken under the same settings as test images. Isotypes controls showed didn't show immunoreactivity to CD-63 or to VAMP-2 antibodies in both cell lines, KU-812 and LAD-2.

We repeated the same set of experiments with VAMP-7 in resting human basophils (Fig. 3.10). As described above, immunoreactivity for CD63 was measurable with BODIPY-FL antibody (green) (lane 2) and immunoreactivity for VAMP-7 was stained with Rhodamine-Red-X- antibody (red) (lane 3). It appears that human basophils express distinct intracellular granularity pattern for CD63 and VAMP-7. A combined image of double immunofluorescence (VAMP-7 and CD63) staining of resting human basophils indicated that SNARE-specific antibody VAMP-7 and large granular marker

CD63 recognized the same intracellular compartments. Merged images of VAMP-7 and CD63 resulted in co-localized yellow color (lane 5).

We examined the two cell lines, LAD-2 and KU-812, for immunoreactivity against VAMP-7 and CD-63 (Fig. 3.11 A and B). Lane 1 represents DAPI-nucleus stain and lane 2 represents CD63 immunoreactivity as described above. VAMP-7 (lane 3) showed discrete cytoplasmic immunofluorescence (lane 4). A combined image of double immunofluorescence (VAMP-7 and CD63) staining of KU-812 and LAD-2 showed considerable overlap. As in the previous image of merged immunoreactivity it appears that CD63 showed a more pronounced perinuclear stained in KU-812. Double immunofluorescence in LAD-2 was similar to human basophils and KU-812 and exhibited intracellular co-localization of CD63 with VAMP-7.

3.4.4.2 Double staining of IL-4 and SNARE-specific antibodies

SNARE isoforms are likely to be important in the release of vesicular products including the cytokines, IL-4 and IL-13. We, therefore, investigated intracellular co-localization of IL-4 with two isoforms of v-SNAREs. We focused our study on VAMP-7 and VAMP-8. Representative images of human basophils were stained with BODIPY-FL-conjugated secondary antibody (green) to detect immunoreactivity against IL-4 (Fig. 3.12) and Rhodamine-Red-X-conjugated secondary antibody (red) to detect VAMP-7 immunoreactivity. We examined over 70 slides of human basophils with average of 20 basophils per slide. Single stain for IL-4 showed two patterns of immunoreactivity. The first pattern showed that IL-4 was scattered throughout the cytoplasm with discrete pinpointed distribution. This pattern of IL-4 staining was present over 80% of the time. The second type of distribution of IL-4 was localized to areas of increase immunoreactivity beyond the punctuate distribution of IL-4. We defined those areas as "IL-4 complexes" (white arrows). Such complexes may represent the granule-vesicle attachment (GVA) units described by Dvorak[28, 29] and they were present in 20% of the cells. Isotype controls for IL-4 did not show any non-specific binding, indicating that those GVA complexes were specifically recognized by IL-4 antibody. Resting peripheral blood basophils labeled with BODIPY-FL indicated that IL-4 immunoreactivity was characteristic with a cytoplasmic pinpoint vesicular staining pattern. Immunofluorescence for IL-4 was less intense when compared with CD63 (Fig. 3.8 – 3.11). Localization of VAMP-7 in human basophils (described previously) was distributed uniformly throughout the cytoplasm, suggesting that immunoreactivity might be present on both granule and vesicular compartments. A combined image of double immunofluorescence (IL-4 and VAMP-7) staining of resting basophils (lane 5) showed only partial co-localization of the proteins. Isotype control for both IL-4 and VAMP-7 (mouse IgG_1, 12.5 μg/ml) were negative (Fig.3.12 IC).

We examined immunoreactivity of resting human basophils stained with BODIPY-FL antibody to detect IL-4 (Fig.3.13) and Rhodamine-Red antibody to detect VAMP-8. Immunoreactivity against VAMP-8 was less pronounced, when compared with VAMP-7 (Fig.3.12). VAMP-8 had the same fine and punctuate pattern and cytoplasmic distribution as VAMP-7. Interestingly, VAMP-8 also had areas of increased fluorescence (yellow arrows). A merged image of stained resting basophils (IL-4 and VAMP-7) (lane 5) exhibited partial co-localization. Areas with increased immunofluorescence did not necessarily coincide in the merged images. Further research is required to define the morphology and functions of those increased areas of fluorescence. An appropriately matched isotype control for both IL-4 and VAMP-8 (mouse IgG_1 (12.5 μg/ml) for IL-4 and rabbit IgG (10 μg/ml) for VAMP-8) and showed consistently negative staining (Fig.3.13 IC).

We acquired high magnification images to evaluate the distribution of IL-4 in LAD-2 mast cells (Fig. 3.14). As described above, IL-4 was randomly distributed throughout the cytoplasm with discrete pinpoint distribution. Immunoreactivity for VAMP-7 was more pronounced when compared with IL-4. On the merged images, it appeared that IL-4 and VAMP-7 had only limited co-localization.

Q-SNARE SNAP-23 was identified on the plasma membranes[30, 31]. We obtained images of human basophils with immunoreactivity against IL-4 (green) and SNAP-23 (red) (Fig.3.15). Intracellular distribution of SNAP-23 showed no distinct granularity when compared with VAMP-2 (Fig. 3.8), VAMP-7 (Fig. 3.10, 3.12), VAMP-8 (Fig. 3.13). SNAP-23 was present both cytosolically and near the plasma membrane. Distribution of SNAP-23 in the cell was distinct from other R-SNARE proteins (Fig. 3.8-3.13) with lack of granularity. Highest fluorescence was observed around the periphery of the cell (yellow arrows) with decreasing immunofluorescence to the center of the cell. SNAP-23 partially covered the nucleus, which may be due to overlying of the plasma membrane over the nucleus during cytospins preparation ("pancake effect"). However, the majority of SNAP-23 had a peripheral distribution. A combined image of double immunofluorescence (IL-4 and SNAP-23) staining of resting basophils (lane 5) showed only marginal co-localization of the proteins. Isotype controls for both IL-4 and SNAP-23 were consistently negative (Fig.3.15 IC).

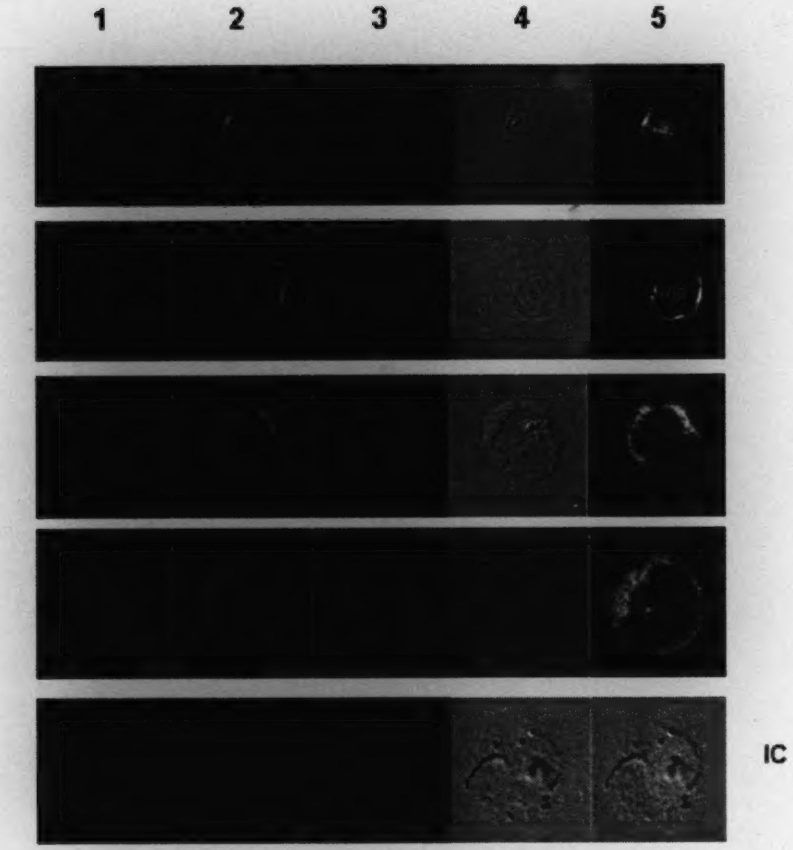

Figure 3-8 Co-localization of CD63 and VAMP-2 in blood basophils.

Lane 1 represents DAPI - nucleus stain. Lane 2: BODIPY-FL-conjugated (BP) secondary antibody (green) to detect immunoreactivity against CD63. Lane 3 represents Rhodamine-Red-X-conjugated (RR) secondary antibody (red) to detect VAMP-2 immunoreactivity. Lane 4 is DIC (Differential Interference Contrast). Lane 5 represents the merged image of VAMP-2 and CD63. IC – isotype control: primary antibody (CD63) was replaced by an irrelevant mouse IgG_1 or secondary antibody to VAMP-2 was replaces with IgG1. No non-specific binding was detected. It appears that significant portion of cytoplasm expresses immunoreactivity for CD63 and VAMP-2, which might be associated with cytoplasmic granules. Immunofluorescence images demonstrated substantial co-localization of VAMP-2 and $CD63^+$. Original magnification is × 600 for all images.

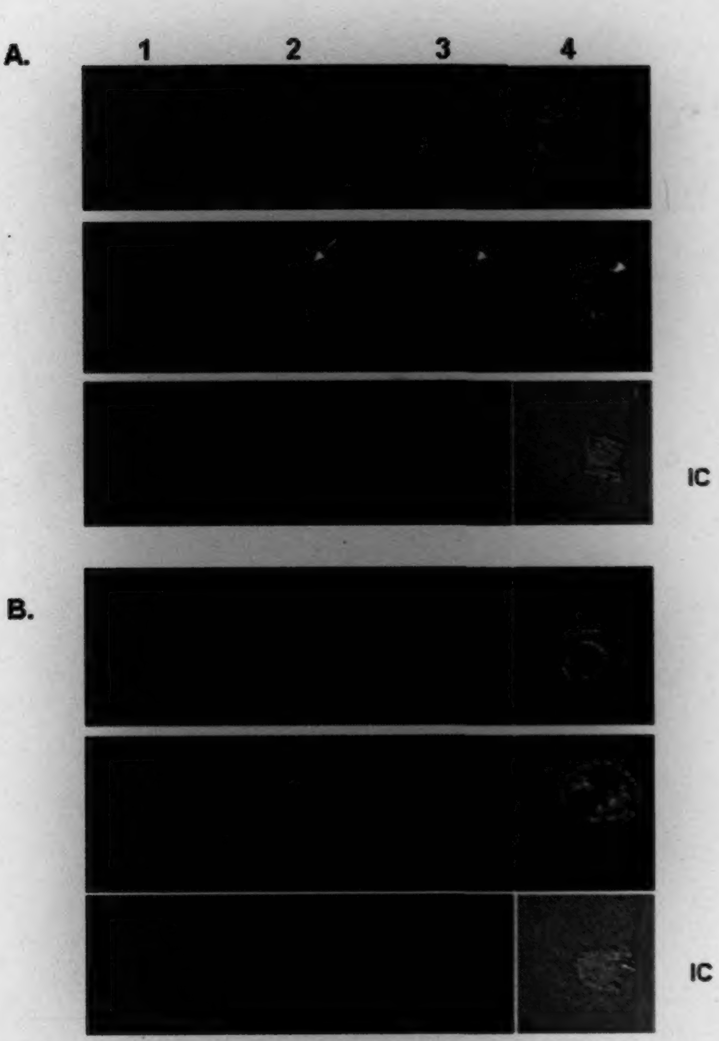

Figure 3-9 Co-localization CD63 and VAMP-2 in immunostained KU-812 (A) and LAD-2 (B).

Lane 1: DAPI - nucleus stain. Lane 2: BP-conjugated secondary antibody (green) to detect CD63. Lane 3: RR secondary antibody (red) to detect VAMP-2. Lane 4: DIC. Lane 5: merged image of VAMP-2 and CD63. IC – isotype control: IgG1/BP//IgG1/RR. No non-specific binding was detected. Two types of immunoreactivity were detected in KU-812: scattered cytoplasmic distribution and focused perinuclear staining (yellow arrow). LAD-2 appeared to have uniform distribution of CD63 and VAMP-2 throughout the cytoplasm.

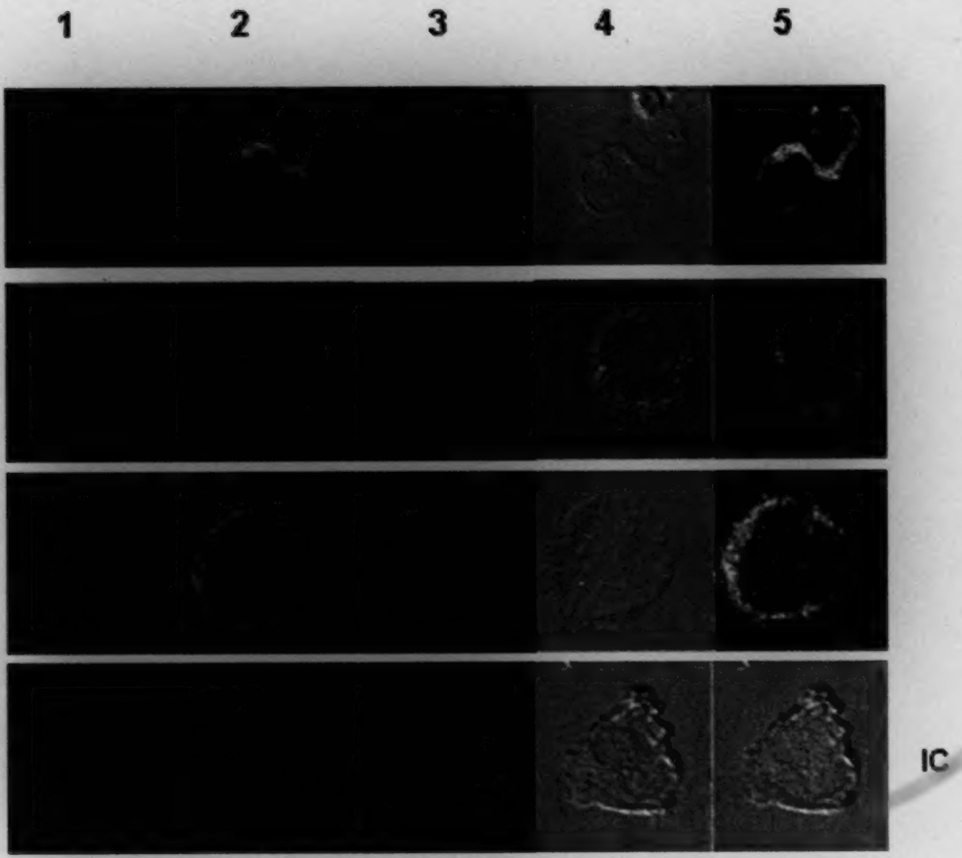

Figure 3-10 Co-localization CD63 and VAMP-7 in immunostained blood basophils.

Lane 1: DAPI - nucleus stain. Lane 2: BP conjugated secondary Ab (green) to detect CD63. Lane 3: RR conjugated secondary antibody (red) to detect VAMP-7. Lane 4: DIC. Lane 5: merged image of VAMP-7 and CD63. IC – isotype control: IgG1/BP//IgG1/RR. No non-specific binding was detected. It appears that human basophils express distinct cytoplasmic granularity pattern for CD63 and VAMP-7. A combined image of double immunofluorescence (VAMP-7 and CD63) staining of resting human basophils indicated that SNARE-specific antibody VAMP-7 and large granular marker CD63 recognized the same intracellular compartments. Merged images of VAMP-7 and CD63 coincided in yellow color.

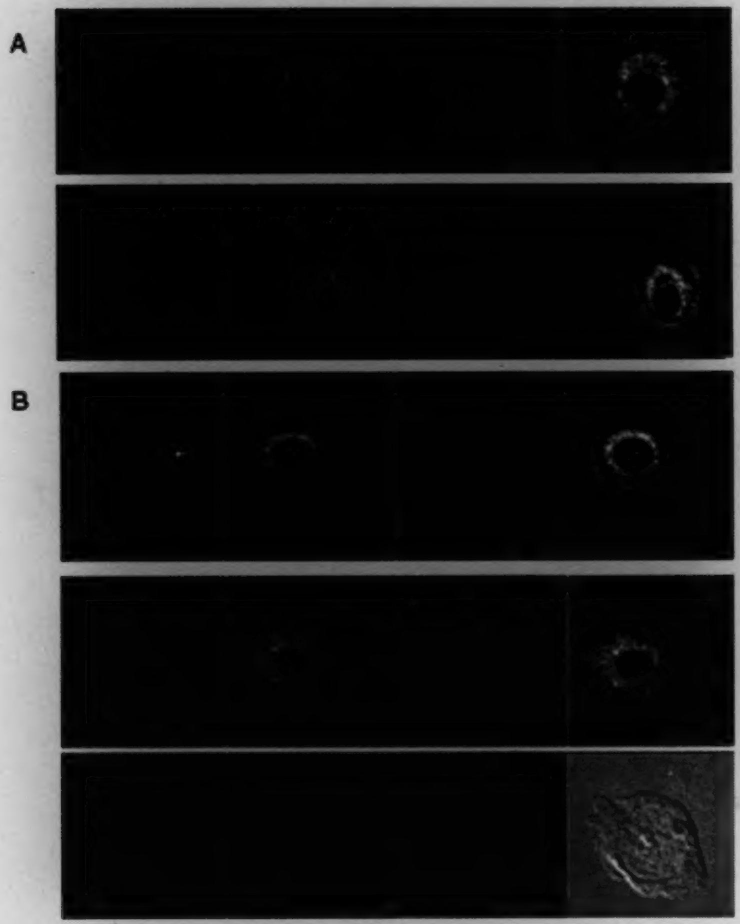

Figure 3-11 Co-localization CD63 and VAMP-7 in immunostained KU-812 (A) and LAD-2 (B).

Lane 1: DAPI - nucleus stain. Lane 2: BP-conjugated secondary antibody (green) to detect CD63. Lane 3: RR secondary antibody (red) to detect VAMP-7. Lane 4: DIC. Lane 5: merged image of VAMP-7 and CD63. IC – isotype control: IgG1/BP//IgG1/RR. No non-specific binding was detected. Immunoreactivity for VAMP-7 in both KU-812 and LAD-2 had mainly perinuclear staining. Only small amount of VAMP-7 was present on the periphery of the cells. A combined images of CD63 and VAMP-7 in KU-812 and LAD-2 detected considerable overlapping, indicating the similar origin for immunoreactivity.

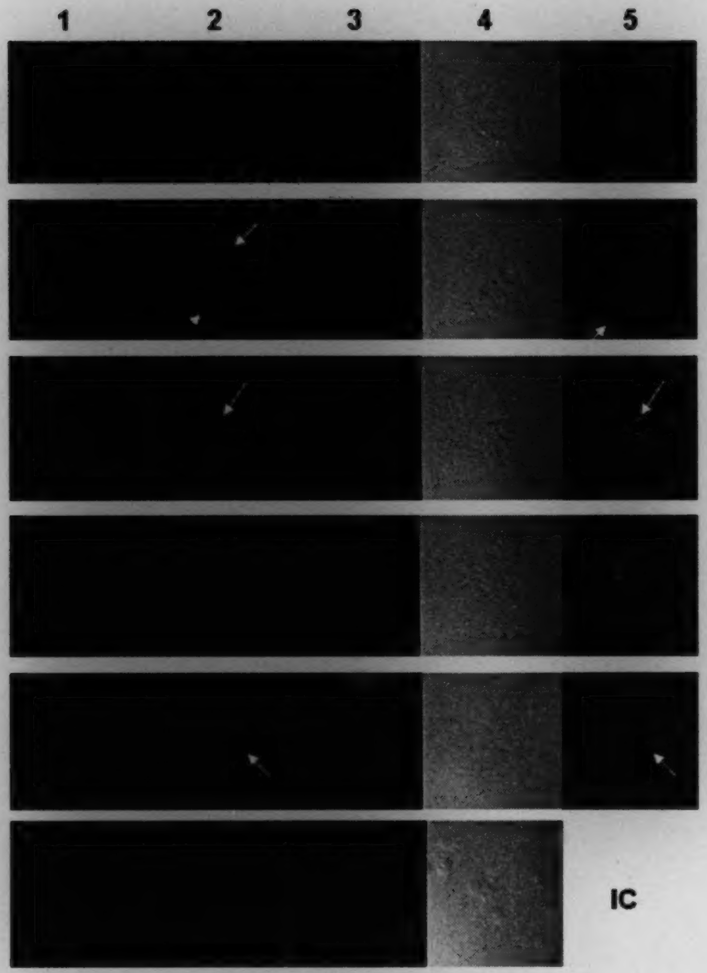

Figure 3-12 Co-localization of cytokine IL-4 and VAMP-7 in human basophils.

Lane 1: DAPI - nucleus stain. Lane 2: BP-conjugated secondary antibody (green) to detect IL-4. Lane 3: RR secondary antibody (red) to detect VAMP-7. Lane 4: DIC. Lane 5: merged image of VAMP-7 and IL-4. IC – isotype control: IgG1/BP//IgG1/RR. No non-specific binding was detected. Intracellular IL-4 showed two patterns of immunoreactivity: scattered throughout the cytoplasm with discrete pinpointed distribution and localized areas of increase immunoreactivity (white arrows). VAMP-7 was uniformly distributed in the cytoplasm. Combined images of IL-4 and VAMP-7 in human basophils showed only partial co-localization of those proteins.

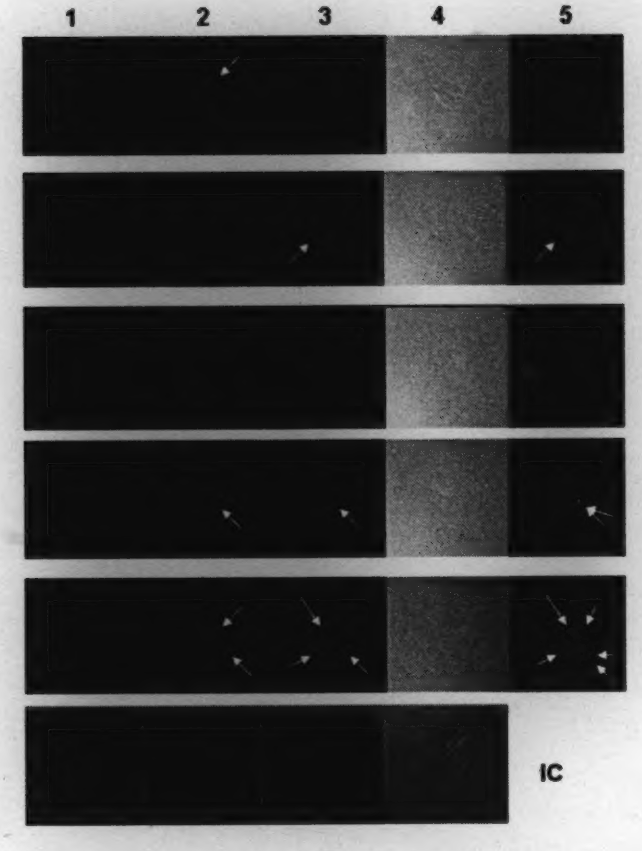

Figure 3-13 Co-localization of cytokine IL-4 and VAMP-8 in human basophils.

Lane 1: DAPI - nucleus stain. Lane 2: BP-conjugated secondary antibody (green) to detect IL-4. Lane 3: RR secondary antibody (red) to detect VAMP-8. Lane 4: DIC. Lane 5: merged image of VAMP-8 and IL-4. IC – isotype control: IgG1/BP//IgG1/RR. No non-specific binding was detected. Lane 5 represents the merged image of VAMP-8 and IL-4. VAMP-8 had an increased fluorescence peaks (yellow arrows) in the cytoplasm of the cells. White arrows represent the areas of increased IL-4 immunofluorescence (explanations in the text). A merged image of double immunofluorescence (IL-4 and VAMP-8) staining of resting basophils showed (1) only partial co-localization of the proteins, (2) Areas with increased immunofluorescence did not necessarily coincided in the merged images (merged arrows vs separated arrows).

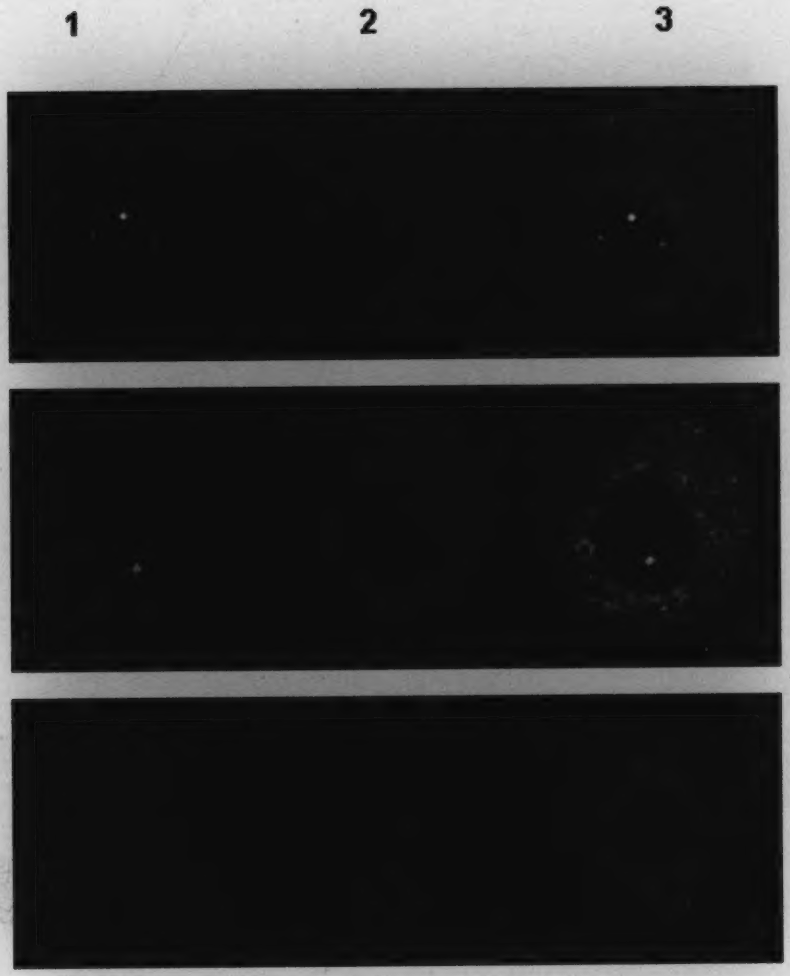

Figure 3-14 High resolution of IL-4 and VAMP-7 co-localization in LAD-2 MC.

Lane 1: BP-conjugated secondary antibody (green) to detect IL-4. Lane 2: RR secondary antibody (red) to detect VAMP-7. Lane 3: merged image of VAMP-7 and IL-4. LAD-2 mast cells showed very limited co-localization of VAMP-7 with IL-4.

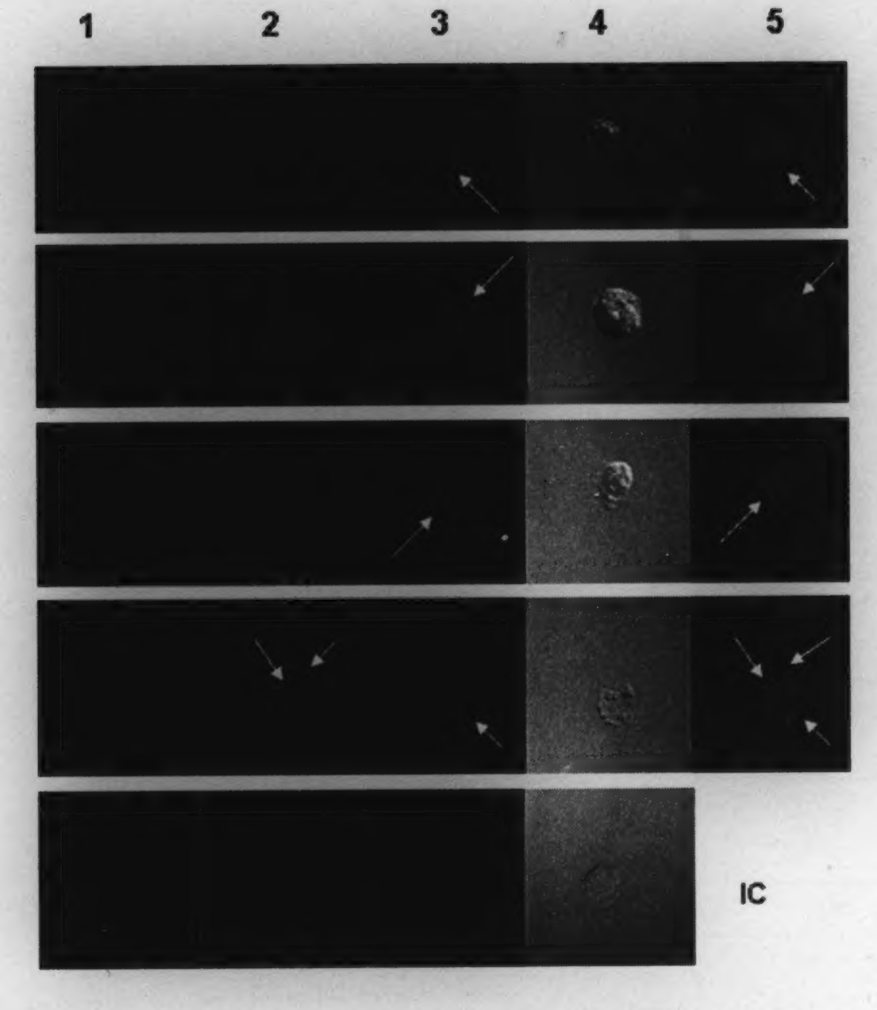

Figure 3-15 Co-localization of cytokine IL-4 and SNAP-23 in human basophils.

Lane 1: DAPI - nucleus stain. Lane 2: BP-conjugated secondary antibody (green) to detect IL-4. Lane 3: RR secondary antibody (red) to detect SNAP-23. Lane 4: DIC. Lane 5: merged image of SNAP-23 and IL-4. IC – isotype control: IgG1/BP//IgG1/RR. No non-specific binding was detected. Intracellular distribution of SNAP-23 showed no distinct granularity.SNAP-23 was present in the cytoplasm and on the plasma membrane. Highest fluorescence was observed around the periphery of the cell (yellow arrows) with decreasing immunofluorescence to the center of the cell (yellow arrows). A combined image of double immunofluorescence (IL-4 and SNAP-23) staining of resting basophils showed only marginal co-localization of the proteins.

3.4.5 Effect of SNARE-specific antibodies on mediator secretion in SLO-permeabilized cells.

We investigated the role of SNAREs in exocytosis by examining secreted cytokines from SLO-permeabilized cells incubated in the presence of isoform-specific SNARE antibodies. Consistent with previous findings[32], we observed that the addition of Ca^{2+} and GTPγS caused a rapid (within 7 min) release of both small vesicles and large granule mediators. Since mature end-differentiated basophils are not easily transfected, we chose to introduce SNARE neutralizing antibodies into permeabilized cells. Our current understanding of exocytosis assumes that human basophils utilized distinct SNARE proteins for small vesicles and large granules mediator release. It is generally accepted that small vesicles contain cytokines, while large granules contain mainly histamine. To confirm the selective involvement of SNARE proteins in human basophils exocytosis, we introduced selected SNARE antibodies against VAMP-7, VAMP-8 and SNAP-23 and measured the mediator release by ELISA.

SLO has been used in studies of Ca^{2+} and GTPγS-mediated exocytosis in human neutrophils[33, 34] and eosinophils (*Logan et al*, in submission) and permits the entry of exogenous proteins, including antibodies, into the cell[35, 36]. It was previously demonstrated that the intensity of the secretory response was dose-dependent on calcium[37]. To be able to detect inhibitory responses using SNARE antibodies we established the maximal secretory response in the presence of an optimal dose of SLO and calcium that would not compromise cell integrity. We utilized a series of EGTA-buffered calcium solutions, to determine the maximal secretion of β-hexosaminidase from the prebasophilic cell line KU-812, as previously described[38] (Chapter 2). Titration of SLO (0.1-1.0 μg/ml) revealed the concentration required to permeabilize KU-812 was

0.1 μg/ml. This concentration is comparable to previously reported effective doses and was used for all subsequent experiments[34, 35, 39]. In control experiments, GTPγS-induced secretory responses were enhanced by addition of Ca^{2+} and reached maximal levels within 7 min at pCa 5 (55% ± 9%) as determined by β-hexosaminidase assay. KU-812 preincubated with isotype antibodies ($mIgG_1$) released 47% of the total β-hexosaminidase. Negative controls (pCa 7) exhibited a small spontaneous release of 11% ± 9% β-hexosaminidase. In separate experiments we determined that KU-812 cells have a spontaneous release (in the absence of any stimulus or Ca^{2+}) of 11.5% ± 5% (data not shown). These observations support the well recognized requirement for Ca^{2+} during exocytosis[38, 40], and indicated that SLO did not compromise granule membrane integrity.

We next tested the effects of SNARE antibodies on basophil secretion using optimal permeabilization and stimulation conditions (Fig. 3.16). Values from separate experiments (n=10) were pooled. Due to the variability among experiments, all data were standardized relative to the control values. Cells alone were defined as 100% release. We used the one-way ANOVA test (Bonferroni's Multiple Comparison Test) to determine the significance between examined conditions. To investigate the significance we use the most stringent setting ($p<0.001$).

To determine the effect of SNARE-specific antibodies we defined the release from non-stimulated cells (cells alone, first column) as 100% and compared the values with the amount of IL-4 released under different conditions. Cells activated with Ca^{2+} and GTPγS are shown in the second column. There is significant increase in IL-4 release compared with non-stimulated cells (143% ± 12%). Cells pre-incubated with isotype antibodies (IgG_1) did not show significant difference compared with cells activated by

Ca^{2+} and GTPγS (121% ± 15%) and cells alone. Cells pre-incubated with VAMP-7 mAb, activated with Ca^{2+} and GTPγS (column four), showed a significant inhibition of IL-4 release (49% ± 18%) compared with matched isotype control (IgG_1). Activated cells (second column) showed significant difference compared to cells preincubated with anti-VAMP-8 antibody (106% ± 9%). No significant difference was detected between activated cells and cells preincubated with anti-SNAP-23 (sixth column) antibody. It is possible that higher doses of anti-SNAP-23 were required to inhibit of mediator release in the cells.

Unstimulated KU-812 cells release negligible amounts of IL-4, however upon activation with *N. americanus* secretory products or house dust mite allergen from *Dermatophagoides pteronysinus* there is an increase in IL-4 production[41]. Our data indicate that KU-812 cells were able to release 104.6 pg/10^6 cells (or approximately 0.10 fg/cell) without additional stimuli and activation. The variation in IL-4 release might be explained by time required to accomplished SLO permebilization (22-30 min). Upon activation, the average secretion of IL-4 was 167.6 pg/10^6 cells (or approximately 0.16 fg/cell). Preincubation with anti-VAMP-7 antibody decreased IL-4 secretion to 72.9 pg/10^6 cells (0.072 fg/cell).

Depending on stimulation, human basophils are capable of releasing various amounts of IL-4. Anti-IgE (10 ng/ml) stimulation of human basophils released 325 ± 73 pg/10^6 of IL-4[42]. There are donor-dependent variations in IL-4 release in human basophils activated with anti-IgE[41]. Marked variations in the IL-4 producing capacity was observed in different temperature conditions: from 282 ± 102 pg/10^6 to 506 ± 87 pg/10^6 [43]. Similarly, activation of human basophils with anti-IgE released 518 ± 123 pg/10^6 of

IL-4, however spontaneous release was only 37.6 ± 26 pg/10^6[44]. Lectins (plant products) can induce IL-4 release from human basophils comparable to IgE activation and compromised 232 ± 82 pg/10^6[45].

Our data provides observations of IL-4 expression in prebasophilic cell line KU-812 and is consistent with observations on IL-4 release from human basophils.

Table 3-1Statistical analysis of one-way ANOVA test (Bonferroni's Multiple Comparison Test) to compare IL-4 release in KU-812 cells

	Cells alone	Cells +SLO+Ca^{2+}	Cells +SLO+Ca^{2+} +IgG1	Cells +SLO+Ca^{2+} +αVAMP-7 Ab	Cells +SLO+Ca^{2+}+ αVAMP-8 Ab	Cells +SLO+Ca^{2+}+ αSNAP-23 Ab
Cells alone	-	**<0.001**	>0.001	**<0.001**	>0.001	>0.001
Cells +SLO+Ca^{2+}	-	-	>0.001	**<0.001**	**<0.001**	>0.001
Cells +SLO+Ca^{2+}+IgG_1	-	-	-	**<0.001**	>0.001	>0.001
Cells +SLO+Ca^{2+}+αVAMP-7 Ab	-	-	-	-	**<0.001**	**<0.001**
Cells +SLO+Ca^{2+}+αVAMP-8 Ab	-	-	-	-	-	>0.001
Cells +SLO+Ca^{2+}+αSNAP-23 Ab	-	-	-	-	-	-

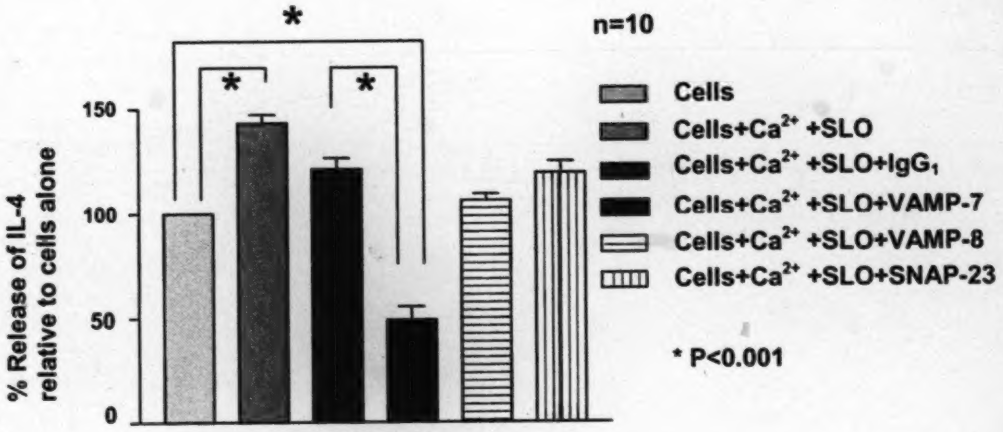

Figure 3-16 Effect of SNARE-specific antibodies on cytokine IL-4 secretion from SLO-permeabilized KU-812.

KU-812 were incubated in the presence of VAMP-7 mAb, VAMP-8 Ab, SNAP-23 Ab or matched concentrations of isotype control antibody (mIgG$_1$), activated with Ca^{2+} and GTPγS for 5 min. Cell supernatants were examined for IL-4 cytokine release as indicated in Materials and Methods. Shown are representative (mean value ± SEM) experiments for an effective dose of 1 μg/ml which exhibited an inhibitory response for these antibodies.

3.5 Discussion

In this study we have demonstrated that human basophils as well as the basophilic cell line, KU-812, express various SNARE isoforms. We found a similar profile of SNARE protein expression in two mast cell lines, suggesting that at least some of the mechanisms of SNARE-dependent granule docking may be common across MC and basophils. Indeed, our findings are similar to previously described observations of SNARE expression proteins in RBL-2H3[46]. Beyond the expression of VAMP-2 and VAMP-7, we have also documented the expression of VAMP-8 as an additional R-SNARE found in RBL cells. A previous study suggested that tetanus-insensitive SNARE isoforms (VAMP-7, VAMP-8), rather than the tetanus toxin sensitive VAMP-2, may be most critical R-SNAREs in the release of histamine and serotonin in this cell type[55]. Thus, the finding of a toxin-insensitive R-SNARE isoform, VAMP-7, in our study may suggest an important role for this protein in human basophil exocytosis. The relative importance of VAMP-7 and VAMP-8 in the release of mediators from different cellular components remains unknown. However, it has been suggested that the release of mediators from rapidly mobilizable small secretory vesicles may be directed by SNARE isoforms distinct from those involved in the release of stored products from dense core granules in human eosinophils[26].

To investigate the potential involvement of various SNARE isoforms in release of mediators from specific cellular compartments, we examined the co-localization of SNARE isoforms with a granule marker, CD63. Basophil granules are regarded as modified secretory lysosomes and, therefore, express CD63 (LAMP-3) (*Dragonetti et al.*, 2000). We showed a strong co-localization of VAMP-7 and VAMP-2 with CD63$^+$

granules in human basophils. This finding suggests that VAMP-7 may be important in the fusion of basophilic granules with the plasma membrane during the release of preformed granule stored products, including histamine. The expression of VAMP-7 reported here is consistent with observations that this R-SNARE is localized to $CD63^+$ compartments in rat basophilic cells (RBL-2H3) and late endosomes/lysosomes in other cell types [47, 48]. Colocalization of VAMP-7 immunoreactivity with $CD63^+$ is a novel observation. Human basophils store pre-formed mediators such as histamine in large granules[7, 49] while cytokines are localized to small secretory vesicles[50]. We were able to demonstrate the co-localization of vesicle-stored product such as IL-4 with three different isoforms of R-SNAREs VAMP-2, VAMP-7 and -8. A small amount of IL-4 was detected in LAD-2 and KU-812 suggesting that those cells might synthesize IL-4 *de novo* upon stimulation and that it is not stored in cytoplasmic compartments. The possibility that the amount of IL-4 produced in the cell lines is considerable smaller in comparison to human basophils cannot be excluded. Pinpoint co-localization of VAMP-7 with IL-4 and CD63 suggest that VAMP-7 might be involved not only in large granular mediator release, but also in mobilization of small vesicles. This is a novel observation and to the best of our knowledge has not been previously described in the literature.

However, it is important to recognize that although confocal laser microscopy can be a powerful tool, it has some serious limitations. Thus, care must be taken in reaching conclusions that are solely based on this technology[51]. The first limitation of confocal microscopy is that the resolution is limited by the wavelength of light. Although confocal microscopy pushes the limit of resolution to the theoretical limit of light microscopy, it does not resolve better then 0.1 micron (under ideal conditions). The size of small vesicles in human basophils varies from 0.1-0.12 microns and large granules from 0.16-0.22 microns[52, 53]. The limit to the resolution of a confocal microscope may cause misleading interpretations of the data when one is using two antibodies to establish whether the structures of interest are separated within the cell. The limit of resolution may lead to the conclusion that they are associated with the same structure (for example secretory vesicles), when in fact they are associated with distinct structures that are not being resolved by the microscope.

To investigate the functional involvement of SNARE proteins in prebasophilic cell line we focused on selected R-SNAREs: VAMP-7 and VAMP-8. The expression of VAMP-7 localized to CD63$^+$ compartments in RBL-2H3 and late endosomes/lysosomes in other cell types[54-56]. Recent studies have revealed intracellular sorting mechanisms of VAMP-7 to CD63$^+$ compartments. Deletion of the amino terminal domain of VAMP-7 (Longin Domain, LD) was shown to cause its mis-sorting from late endosomes to the apical membrane in transfected fibroblasts and inhibit neurite outgrowth[48]. Recent findings support a mechanism that may controlle both localization and function of VAMP-7 through the LD. The LD of SNARE proteins is responsible for the fine-tuning of SNARE function. The LD interacts with a subunit of an adaptor protein which has been demonstrated to be critical for the sorting of membrane proteins such as CD63 (LAMP-3), LAMP-1 and LAMP-2 to lysosomes[57].

We investigated the functional role of SNAREs in exocytosis by examining secreted cytokines from SLO-permeabilized cells incubated in the presence of isoform-specific SNARE antibodies. Our results demonstrate, again for the first time that VAMP-7 is involved in the exocytosis of granule-derived mediators from human basophils. We observed that VAMP-7 mAb, which specifically recognizes the cytoplasmic SNARE-binding domain, inhibit the release of the small vesicles (IL-4) mediators, suggesting that this R-SNARE may be involved in basophils exocytosis. Our observation is in agreement with other studies on permeabilized human neutrophils[33, 34] and eosinophils[38].

Depending on stimulation, human basophils are capable of releasing various amounts of IL-4. Anti-IgE (10 ng/ml) stimulation of human basophils released 325 ± 73 pg/10^6 of IL-4[42]. There are donor-dependent variations in IL-4 release in human basophils activated with anti-IgE[41]. Marked variations in the IL-4 producing capacity was observed in different temperature conditions: from 282 ± 102 pg/10^6 to 506 ± 87 pg/10^6 [43]. Similarly, activation of human basophils with anti-IgE released 518 ± 123 pg/10^6 of IL-4, however spontaneous release was only 37.6 ± 26 pg/10^6 [44]. Lectins (plant products) can induce IL-4 release from human basophils comparable to IgE activation and compromised 232 ± 82 pg/10^6 [45].

Antibody to VAMP-8 in basophils appeared to have a limited effect on release of small secretory vesicles and release of IL-4. However, the accessibility of the VAMP-8 epitope may be limited *in situ,* thus accounting for the ineffectiveness of VAMP-8 Ab treatment. Further studies are required to determine the role of granule-associated VAMP-8 in secretion from these cells. Antibody to SNAP-23 did not affect the release of IL-4 in basophils, suggesting that the higher concentration of antibody is required.

Similar to other inflammatory cells, VAMP-2 was localized to small secretory vesicles and/or large granules in previous studies[23, 58]. Recent studies demonstrated that VAMP-2 mAb impaired the surface upregulation of the common secondary and tertiary granule marker, CD66b, but not the azurophilic granule marker, CD63, in electropermeabilized neutrophils[59].

We identified several Q-SNAREs, SNAP-23, syntaxin-4 and syntaxin-6 that are expressed in human basophils and are capable of interacting with multiple R-SNAREs[19;20;43;44]. According to the SNARE hypothesis, Q-SNARE contains of SNAP-23/syntaxin-4 or -6 *cis*-SNARE complexes, which may limit epitope availability of SNAP-23 during activation, thereby reducing the inhibitory effect of the SNAP-23 mAb on large granules. Our results further showed similar expression of each SNARE isoform examined in human basophils and MC. Whether SNARE isoforms in basophils and MC employ the same or different cognate partners in the two cells types, leading to identical or divergent pathways of exocytosis, remains to be determined.

In conclusion, we propose that human basophils express a range of SNARE isoforms likely to be important for docking during the exocytotic process leading to mediator secretion. Our study indicated that VAMP-7 is a promising candidate for targeting the inhibition of release from human basophils. Our observation that VAMP-7 is involved in mediator release from different granule/vesicle compartments is consistent with recent observations that these SNAREs molecules are capable of interacting with multiple partners, and have the potential to participate in multiple membrane fusion events[60, 61]. Involvement of other SNARE proteins in trafficking of histamine-containing granules and secretory vesicles in human basophils exocytosis remains to be determined. Our findings also support the notion that targeting SNARE isoforms with novel pharmaceuticals aimed at inhibiting mediator release maybe an important and viable therapeutic strategy in inflammatory conditions such as asthma and allergic reactions.

Blocking SNARE activity may prevent tissue damage and provide better disease management.

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4 CHAPTER

4.1 GENERAL DISCUSSION AND CONCLUSIONS

4.1.1 Discussion

In this study we have described the expression of SNARE proteins in human basophils and a basophilic cell line KU-812 and mast cell line LAD-2, using RT-PCR, western blotting and immunocytochemical and confocal techniques. Both Q- and R-SNAREs are expressed in these cells. Co-localization studies using specific SNARE-specific antibodies and basophil granule markers showed that CD63 co-localized with VAMP-2, VAMP-7 and VAMP-8. Immunoreactivity against VAMP-7 and IL-4 and VAMP-8 and IL-4 showed only marginal co-localization. We investigated the functional role of SNAREs in exocytosis by examining secreted cytokines from SLO-permeabilized cells incubated in the presence of isoform-specific SNARE antibodies. Our results demonstrate for the first time that VAMP-7 is most likely involved in exocytosis of granule-derived mediators from human basophils.

4.1.1.1 R-SNAREs - their potential role in granule/vesicle trafficking.

Our results agree with previous studies showing the expression of R-SNAREs in haematopoietic cells. *Brumell et al* reported the identification of VAMP-2, assessed by immunoblot analysis using specific antibodies in neutrophils[1]. VAMP-2 was concentrated mainly in tertiary and secretory granules but not on azurophilic and specific granules. Similarly, studies by *Blank et al.* and others have shown that R-SNARE proteins are expressed in MC[2], epithelial cells[3, 4], platelets[5, 6]. Our lab has shown that VAMP-2 was expressed in membrane-enriched fractions with no detectable immunoreactivity in crystalloid granule-enriched fractions from human eosinophils[7]. In our study we demonstrated that VAMP-2, VAMP-7 and VAMP-8 co-localized to large CD63$^+$ granules and to a lesser extend to secretory vesicles.

Unlike other SNAREs, which have been widely described in hematopoietic cells, VAMP-7 has only been shown in neuronal cells and insulin-secreting beta pancreatic cells. In addition, while most VAMPs are cleaved by tetanus neurotoxin (TeNT), VAMP-7 is insensitive to this toxin. Thus, recent studies have shown that this toxin-insensitive VAMP has significant structural differences when compared with other VAMP isoforms. Recent studies by *Galli* demonstrated that TeNT-resistant exocytotic events required for the elongation of dendrites and axons are critically dependent on VAMP-7 during nerve development[8]. Whether suchstructural uniqueness translates to functional differences is currently unknown. In the nervous system, VAMP-7 was shown to be very important in axon outgrowth in rat brain[9] and lipid rafting in canine kidney cells[10]. Similarly, VAMP-7 was recently shown to play a significant role in granule exocytosis in both neutrophils and eosinophils (*Logan*, in submission). Interestingly, VAMP-7 immunoreactivity in developing neurons does not coincide with VAMP-2$^+$ compartments, supporting the notion that distinct sets of R-SNAREs mediate vesicle/granule trafficking[11].

In this study, I have investigated the potential role of SNARE proteins in basophil exocytosis. Upon stimulation human basophils and mast cells may release granular meditors such as histamine, heparin and chymase over 1-5 minutes. Small vesicles contain cytokines such as IL-4, IL-6, IL-8, IL-10, IL-13, and TNF and TGF-β. In accordance with the SNARE hypothesis[12], we predicted a potential role for SNARE isoforms in exocytosis of stored mediators from human basophils and MC. We, therefore, investigated whether SNARE proteins have any role in the release of mediators from both compartments.

From previous studies on human eosinophils and neutrophils in our group, the intracellular application of Ca^{2+} and GTPγS into SLO-permeabilized granulocytes was an effective, but artificial method to induce secretion from these cell types[13-15]. The SLO-permeabilization technique, used for the studies described in chapter 2, is an effective method to introduce neutralizing antibodies into the cell interior. This technique was found to be superior to the electroporation protocol (voltage 600V with set pulse length 99μsec), which is limited by the low viability of cells (less then 45%) and slow re-sealing rate following permeabilization. Although the technique of SLO-permeabilization has its limitations, particularly due to the disruption of cytoskeleton and transduction of signaling with subsequent partial loss of cell responsiveness following permeabilization, it is a useful tool to determine the functional role of candidate proteins involved in exocytosis using neutralizing antibodies. Terminally-differentiated cells are almost impossible to transfect, the latter being an alternative approach to interfering with exocytosis process.

We modified the SLO-permeabilization technique and standardized it for use in human basophils to make it applicable to this cell type and the low number of basophils routinely obtained following isolation from human peripheral blood (see Appendix, chapter 4). Consistent with previous findings, we observed that addition of Ca^{2+} and GTPγS caused a rapid release of both small vesicles (IL-4) and large granule (β-hexozaminidase) release from human basophils. We, subsequently, examined exocytosis of small secretory vesicles from basophils and used specific antibodies to inhibit mediator release. Secretory vesicles contain de novo synthesized cytokines such as IL- 4, IL-6, and IL-13, which can be measured in the supernatant following stimulation.

Previous studies have shown that CD63 upregulation is a reproducible and reliable marker of exocytosis in haematopoietic cells[16]. We attempted to measure upregulation of CD63 expression in permeabilized and activated basophils upon introduction of SNARE-specific antibodies. However, due to specifics of SLO-permeabilization certain percent of the cells remained to be unsealed. It makes it difficult to measure the degranulation, if CD63 can be detected both intracellularly and on the surface of the cell. In contrast to human eosinophils, we found that human basophils abundantly expressed VAMP-2, VAMP-7 and VAMP-8 on the CD63$^+$ crystalloid granules. It is likely that these R-SNAREs maybe involved in granule release in human basophils.

Our studies indicated that treatment with anti-VAMP-7 antibody, but not VAMP-8 inhibited the release of IL-4 in KU-812. This observation further confirms the suggestion that cytokines may be differentially released from intracellular compartments. This is similar to the observation in the eosinophil where SNARE proteins were differentially involved in mediator release.

The data generated here by our team support a promising role for VAMP-7 in exocytosis of large and small granules from human basophils. In contrast, VAMP-2 and VAMP-8 are also present on small vesicles as well as on large granules. However, they do not appear to play a significant role in exocytosis. The role of these R-SNAREs requires further examination to determine their precise functional involvement. The Q-

SNAREs, SNAP-23 and syntaxin-6 have been identified as a potentail docking partners for VAMP-2, VAMP-7 and VAMP-8 for exocytosis in basophils.

We also focused study on selected tetanus-insensitive R-SNAREs: VAMP-7 and VAMP-8. The R-SNARE can be subdivided into short VAMPs or "brevins" and long VAMPs or "longins", on the basis of whether they contain a short and variable domain or a conserved long domain of 120-140 amino acids at their N-terminus[17].

Recent studies by *Galli* have provided sufficient evidence that VAMP-7 is critically important in the maturatiom of axon terminals during brain development of rats[9, 18, 19]. It has previously been reported that VAMP-7 was localized in the somatodendritic compartment of neurons indicating a role in membrane fusion events within dendrites. There are a sufficient number of studies demonstrating that R-SNARE is also involved in vesicle release from non-neuronal tissues[20-22]. Recent studies have revealed an intacellular mechanism of sorting VAMP-7 to CD63$^+$ compartments. Deletion of the amino terminal domain of VAMP-7 (longin domain) was shown to cause its mis-sorting from late endosomes to the apical membrane in neurite outgrowth[23]. Recent findings also support a mechanism controlling both localization and function of VAMP-7 through the LD and clathrin adaptors. The LD was demonstrated to interact with a subunit of an adaptor protein which has been demonstrated to be critical for the sorting of membrane proteins such as CD63 (LAMP-3), LAMP-1 and LAMP-2 to lysosomes[24]

Preliminary data from our laboratory has indicated that, in addition to basophils, eosinophils and neutrophils, other cell types express VAMP-7. These include the antigen-specific cytotoxic lymphocyte (CTL) clones and the human NK cell line, YT Indy. Each of these cell types also express protein for R-SNAREs VAMP-7 and VAMP-8 and Q-SNAREs SNAP-23, syntaxin-4 and -6. Further studies are currently underway to determine their functional role in the release of granule-stored mediators.

4.1.1.2 Q-SNAREs - potential partners

To date, 16 mammalian syntaxin family members have been identified, all of which localize to specific membrane compartments along the exocytic and endocytic pathways. The first group of syntaxins identified (syntaxins 1-4) are predominantly restricted to the plasma membrane where they mediate constitutive and regulated vesicle trafficking events at the cell surface[26-28] In contrast, syntaxins 5, 6, 10, 11, and 16 are localized to different subcompartments within the Golgi apparatus[29, 30], whereas syntaxins 7, 12, and 13 are found in the post-Golgi endosomal population[31-33]. In our study we were able to detect mRNA and protein encoding for several Q-SNAREs: SNAP-23, syntaxin-4 and syntaxin-6. Those proteins have been shown to interact with multiple R-SNAREs[23, 34, 35]. It was shown that SNAP-23, syntaxin-4 and VAMP-2 are critical docking partners for assembly of *trans*-SNARE complexes (between two membranes) during granule/vesicle docking. In addition, *cis*-SNARE complexes (on the same membrane) have been reported for Q-SNAREs which may be important for the regulation of SNARE assembly[2] (Figure 4.1).

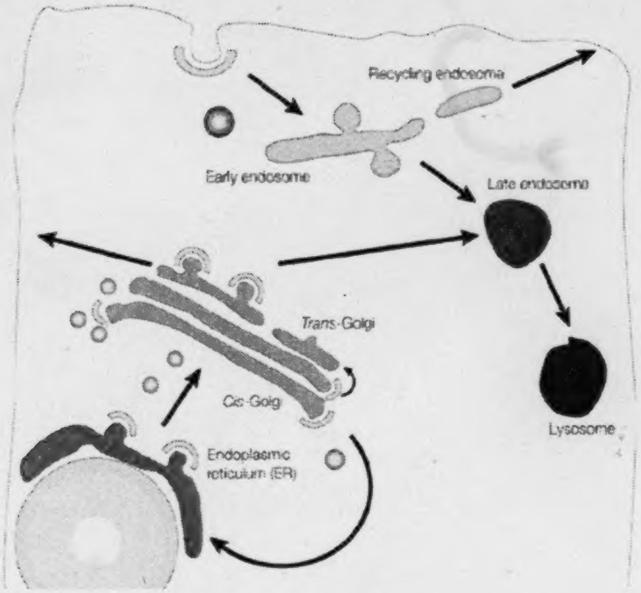
Recycling endosome
Early endosome
Late endosome
Trans-Golgi
Cis-Golgi
Lysosome
Endoplasmic reticulum (ER)

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Figure 4-1 The major membrane traffic pathways.
In the biosynthetic pathway, newly synthesized molecules are transported from the endoplasmic reticulum to the Golgi and from one cisterna of the Golgi to the next until they reach the *trans*-Golgi network. There, sorting occurs, directing traffic to the plasma membrane or to endosomes. In the endocytic pathway, macromolecules are internalized at the plasma membrane and forwarded to early endosomes, from where they are either recycled to the plasma membrane through recycling endosomes or forwarded towards degradation in late endosomes and lysosomes.

Adopted from: Tomas Kirchhausen, Nature Reviews Molecular Cell Biology 1; 187-198 (2000)

Recent studies indicate SNAP-23 and syntaxin-4 are critical components of the exocytosic machinery in mast cells, eosinophils[36, 37], mast cells[2, 38, 39], platelets[40, 41] and neutrophils[25, 42, 43]. It was postulated that both SNAP-23 and syntaxin-4 are exclusively localized to plasma membrane. However, in addition with plasma membrane localization, SNAP-23 and syntaxin-11 were detected in late endosomes and the trans-Golgi network in HeLa cells suggesting that those proteins play a role in vesicle trafficking. A previous report in our lab showed the similar localization of SNAP-23 and syntaxin-4 in human eosinophils[36]. In agreement with those observations, we were able to detect SNAP-23 localized predominantly to the cell periphery with only punctate cytoplasmic staining. Immunoreactivity against SNAP-23 in cytoplasm may suggest that this protein is also involved in granule/vesicle shuttling from Golgi to ER.

Our data showed that SNAP-23 is mainly present in the plasma membrane. For SNARE complex to be efficient SNAP-23 should interact with another Q-SNARE such as syntaxin-4 or syntaxin-6 and one of the R-SNAREs. Thus, it is likely that the mobilization of $CD63^+$ specific basophil granules and $IL\text{-}4^+$ vesicles is dependent on the use of syntaxin-4 or-6 as Q-SNAREs on the plasma membrane. Our data is in agreement with previous data showing differential mobilization of cytokines from intracellular compartments of human eosinophils[44, 45]. It is, however, possible that syntaxin-6 is cognate cis-SNARE partner for SNAP-23 on the plasma membrane.

4.1.2 Summary and proposed model of human basophils exocytosis

Exocytosis occurs by a highly regulated series of events. These include mobilization or translocation of the vesicle/granule to the cell periphery, tethering of the granule to the plasma membrane and docking between the vesicle/granule and plasma membrane. These are critically important events leading to membrane fusion and mediator release[5]. I have already outlined several patterns of exocytosis (Chapter 1) with special attention on PMD. This pattern of mediator release of is thought to occur through small vesicles trafficking to the plasma membrane leading to secretion, apparently independently of large granule events. Previous studies showed that tissue basophils progressively secrete granule contents over several days. It was shown that a large number of small cytoplasmic vesicles participate in this process[46]. These vesicles often were seen to be concentrated in perigranular and peripheral areas.

Although PMD has been described in human basophils, there is no compelling evidence to confirm granule-vesicle fusions or complex Golgi budding by methods other than electron microscopy[46-48]. Extensive work was done by *Dvorak* to characterize the degranulation properties of human basophils. The ultrastructural morphology of PMD has been well defined within a general degranulation model for basophils in 1975. PMD involves the mobilization and secretion of small vesicles independent of large granule events and is implicated in the selective release of stored mediators. Similar to eosinophils, the CLC protein was localized to the cytosol and in the euchromatin of the nucleus[49, 50]. CLC protein-containing granules were found more frequently in basophils than in eosinophils[49, 50]. There are two distinct populations of granules in human basophils. Those that are CLC positive and CLC negative. In experiments with double-labeling (CD63 is a marker for histamine-containing granules and CLC) both granule populations were highly labeled for CD63 suggesting both belong to the histamine-containing granules in basophils[49]. Small secretory vesicles do not express CD63 on their surface. Moreover, small secretory vesicles contain cytokines (IL-4 and IL-13) and albumin, whereas large granules do not. In our study, we have shown that CD63$^+$ granules coincide with VAMP-2, VAMP-7 and VAMP-8 (Fig.4.2).

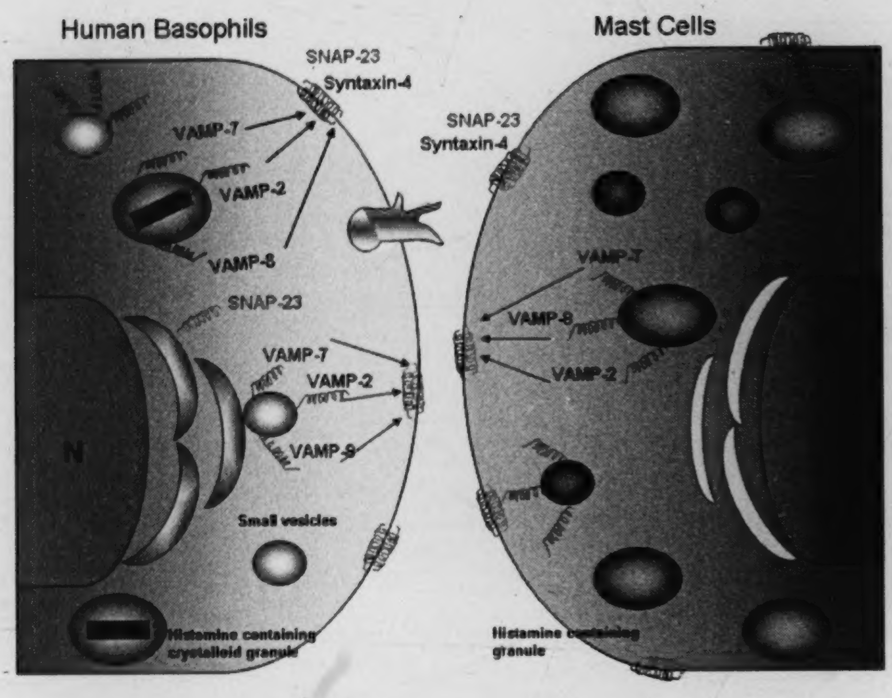
Human Basophils
Mast Cells
SNAP-23
Syntaxin-4
VAMP-7
VAMP-2
VAMP-8
SNAP-23
VAMP-7
VAMP-2
VAMP-8
N
Small vesicles
Histamine containing crystalloid granule
SNAP-23
Syntaxin-4
VAMP-7
VAMP-8
VAMP-2
Histamine containing granule

Figure 4-2 Schematic representation of SNARE isoforms in human basophils and mast cells exocytosis.

The R-SNARE, VAMP-2, -8 and VAMP-7 were localized to small vesicles and crystalloid histamine-containing granules (basophils) and large granules in mast cells. The Q-SNAREs, SNAP-23 were demonstrated to be the docking partners for VAMP-8 and VAMP-7 and they were mainly localized to the plasma membrane. Small amount of SNAP-23 were present on cytoplasmic compartments (endoplasmic reticulum). VAMP-7 was present on both large granules and small vesicles. Antibody mediated inhibition of VAMP-7 support the notion that this protein is involved in release of histamine-containing granules and IL-4 containing vesicles. Anti-VAMP-8 treatment did not show the inhibitory effect on mediator release.

One would anticipate that during the budding of the secretory vesicles from histamine-containing granules vesicles should contain shared membrane proteins. Our studies suggest the presence of such shared spectrum of R-SNAREs between secretory vesicles and large. A useful tool to discriminate the granules and vesicles population is to perform subcellular fractionation of human basophils together with SNARE-specific antibodies. However given the difficulties of generating a sufficient number of human basophils (a minimum of 50×10^6 purified cells per preparation is required), subcellular fractionation is not a viable option at this time. Another approach that would facilitate research into intracellular trafficking of granule/vesicle population is immunocytochemistry of resting and activated human basophils using different stimuli. Recent data on human basophils indicate that basophils react differently upon stimulation with various stimuli. It was shown that activation of human basophils with IL-3 leads to the release of a large amount of IL-4 while releasing a comparatively lesser amount of histamine[51]. Conversely, stimulation with fMLP leads to the release of a high quantity of histamine and a low amount of IL-4, suggesting that IL-4 and histamine may not be stored in the same cellular compartment[51].

Small vesicles in rested basophils are distributed throughout the cytoplasm and are readily mobilized to the cell periphery following cell activation (anti-IgE, fMLP)[46, 47]. In our study we were able to detect immunoreactivity for IL-4 in rested basophils. IL-4 had a pinpoint cytoplasmic distribution. It appeared that IL-4 immunoreactivity exhibited marginal co-localization with VAMP-7 and VAMP-8. There is evidence to suggest that human basophils have the capacity to store IL-4 in secretory vesicles as well as generate it *de-novo*[52]. However, it is presently unknown if these compartments are similar to the stored small vesicle pool in unstimulated cells or may represent a new post-Golgi-derived vesicle population. Small vesicles from human basophils contain albumin, indicating an endocytic origin. It appears that SNAREs localized to small vesicles maybe involved in endocytic recycling. Exocytosis of VAMP-7 vesicles has been observed in epithelial cells[8], insulin producing beta cells[22] and developing neurons[9, 53] indicating that VAMP-7 may be a candidate R-SNARE for small vesicle exocytosis.

Since membrane proteins are thought to be transported along the secretory pathway, a given protein may transiently occupy several compartments en route to its final destination. Indeed, delivery of proteins to various intracellular destinations may in some cases require a series of sorting decisions. One such critical sorting step occurs at the *trans*-Golgi network where proteins with specific targeting signals are incorporated into vesicles with defined trafficking destinations[54, 55]. In contrast, membrane proteins without specific sorting signals are transported along the entire secretory pathway and accumulate at the plasma membrane under steady-state conditions[56]. Our understanding of vesicular trafficking is largely based on detailed studies of endoplasmic reticulum to Golgi trafficking and in the docking and fusion of synaptic vesicles with the presynaptic membrane[57]. According to the SNARE hypothesis that postulate that interactions between R- and Q-SNARE are an important step that confers specificity to the trafficking of different granule/vesicle populations to their target membrane[58, 59]. However, analysis of specific R- and Q-SNARE binding interactions demonstrates a high degree of promiscuity, at least *in vitro* [59, 60]. In other words, the specificity of membrane fusion is not dictated by the interactions between SNAREs.

4.1.3 SNARE isoforms involved in other hematopoietic cells

The first implication of the SNARE proteins in non-neuronal issues was published over a decade years ago. Since that time a number of studies, as well as observations from our laboratory, have demonstrated a critical role for SNAREs in granule/vesicle trafficking in other hematopoietic cell types. Below is a brief overview of isoforms that have been implicated in granule/vesicle exocytosis and docking with the plasma membrane. (Table 4.1)

Cell	Model of exocytosis	Granule(s)	Granule/vesicle		Plasma membrane* Q-SNARE
			R-SNARE(s)	Q-SNARE(s)	
Eosinophils	Classical,	Crystalloid granules	(-) VAMP-2,-7,-8	ND	SNAP-23
	PMD,	Small granules	ND	ND	Syntaxin-4
	Compound	Small vesicles	VAMP-2	ND	
Neutrophils	Classical	Azurophilic granules	(-) VAMP-2,-7,-8	ND	SNAP-23
	Compound	Secondary granules	VAMP-2	SNAP-23, -25	Syntaxin-4
		Tertiary granules	VAMP-2	SNAP-25	Syntaxin-6
		Small vesicles	VAMP-2	ND	
Mast cells	Classical	Secretory granules	VAMP-2,-3,-7,-8	SNAP-23	SNAP-23
	PMD	Small vesicles	VAMP-2,-3,-7	ND	Syntaxin-2,-3,-4
	Compound				
Macrophages	Classical	Small vesicles	VAMP-2,-3,-7,-8	Syntaxin-4	Syntaxin-2,-3,-4,-6
	compound	Phagosomes	VAMP-2,-3,-7,-8	Syntaxin-2,-3,-4	
Basophils	Classical	Crystalloid granules	VAMP-2,-3,-7,-8	SNAP-23	SNAP-23
	PMD	Small vesicles	VAMP-2,-7,-8		Syntaxin-4, -6
	Compound				

Table 4-1 Granule populations and pattern of exocytosis in hematopoietic cells.

(--) denotes absence; ND - not determined

Entries from Q-SNARE(s) in the plasma membrane column are unrelated to the entries in the granule/vesicle membrane.

Modified from: Logan M et al, J Allergy Clin Immunol. 2003 May;111 (5):923-32

4.1.3.1 Mast cells

Our observation on SNARE proteins expression in LAD-2 MC is in agreement with other studies on MC. It has been shown that MC (RBL cells) express multiple syntaxin isoforms including syntaxins 2, 3 and 4 as well as SNAP-23[38]. The distribution of these four proteins has been confirmed biochemically (cell fractionation) and morphologically (immunofluorescence microscopy). SNAP-23 and syntaxin-4 are both located in plasma membrane while syntaxin-3 and VAMP-2 are detected as granular proteins. In resting mast cells, SNAP-23 is concentrated in distinct focal points of lamellipodia-like projections of the cell membrane[38]. We identified four Q-SNARE isoforms in LAD-2 cells: syntaxin-3, -4, -6 and SNAP-23. SNAP-23 located mainly to the plasma membrane as well (confocal microscopy). Previous studies showed, that the plasma membrane-localized SNAP-23 played a role in degranulation as the introduction of anti-SNAP-23 antibodies into permeabilized rat peritoneal MC substantially inhibited the degranulation response[2, 38]. Thus, it is likely that one of the SNARE complexes involved in degranultion contains SNAP-23 and syntaxin-4.

VAMP-2, 3, -8 and VAMP-7 have all been reported to localize to granules and/or small secretory vesicles in the rat basophilic cell line, RBL-2H3[61, 62]. The observation that overexpressed GFP-VAMP-7 colocalized with the granule marker, CD63, may suggest it plays a role in exocytosis, similar to other granulocytes[63]. However, tissue mast cells have not been studied in detail in respect to R-SNARE function, although VAMP-2 was shown to exhibit a punctate intracellular staining pattern in rat peritoneal mast cells (RPMC).

4.1.3.2 Neutrophils

SNAP-23 was found to bind tightly to multiple syntaxins and VAMPs *in vitro*, and played an important role in transport vesicles, docking and fusion in human neutrophils[64]. Soon after, spliced variants of SNAP-23A and SNAP-23B were identified on secondary granules by immunoblot analysis[65]. Parallel with the discovery of SNAP-23, the co-expression of multiple syntaxin isoforms: 1A, 3 4, 5, 6, 7, 9, 11 and 16 mRNA in human neutrophils was reported. [66]

Brumell et al previously showed that VAMP-2 was found mostly in secretory vesicle/plasma membrane enriched fractions and, to a lesser extent, in tertiary granules fractions from human PMNs. VAMP-2 immunoreactivity did not coincide with CD63 and CD67 (now CD66b), but co-localized to CD35, a marker of small vesicles(32). In another study by [36] *Mollinedo et al*, VAMP-2 was localized to some secondary and tertiary granules by immunogold electron microscopy[42]. *Logan et al* showed that VAMP-2 mAb impaired exocytosis of $CD66b^+$ secondary and tertiary granules, but not $CD63^+$ azurophilic granules in electropermeabilized PMNs. The study demonstrated that TeNT was found to impair CD66b surface upregulation, although an inactivated toxin control was not utilized nor CD63 examined[42]. VAMP-2 was concentrated mainly in tertiary and secretory granules but not on azurophilic and specific granules. The observation that antibodies directed to VAMP-7 impaired the secretion of distinct markers of azurophilic, secondary and tertiary granules in permeabilized PMNs supported that VAMP-7 is involved in the trafficking of more than one granule compartment.

4.1.3.3 Eosinophils

The first implications of SNARE proteins in eosinophils came from guinea pig eosinophil studies[67]. It was shown that they expressed protein for SNAP-25, VAMP-1 and VAMP-3. It was demonstrated that VAMP-2 is rapidly mobilized together with RANTES during IFN-γ-induced piecemeal degranulation. The intriguing finding was that eosinophil crystalloid granules were negative for VAMP-2 immunoreactivity and suggests that VAMP-2 may not regulate membrane fusion of these granules with plasma membrane[68]. Eosinophils expressed SNAP-23 and syntaxin-4 both of which were predominantly localized to the plasma membrane[36]. Recent studies in our lab provided compelling evidence that VAMP-7 was localized to $CD63^+$ eosinophil crystalloid granules and was involved in exocytosis of these compartments. VAMP-7 was also expressed in membrane-enriched compartments, indicating it was not exclusively localized to large granules. VAMP-7 was identified to be expressed in human eosinophils and to play a major role in exocytosis of large and small granules. In contrast, VAMP-2 was restricted to small vesicles and is not appear to play a critical role in exocytosis. The Q-SNAREs, SNAP-23 and syntaxin-4 have been identified as docking partners of VAMP-2 and VAMP-7 for eosinophil exocytosis.

4.1.3.4 Platelets

Human platelets express the Q-SNAREs syntaxin-2, -4 and -7, SNAP-23 and SNAP-25[6, 40, 41] and are reported to contain negligible amounts of syntaxin-1, -3 and -5[69] Electron microcscopy studies indicate that SNAP-23, syntaxin-2 and syntaxin-4 are localized to multiple membranes including: storage granules, membrane channels and the plasma membrane[6]. Functional analyses of platelet secretion support that Q-SNAREs are involved in multiple docking events and mediator release. Antibody-mediated inhibition of syntaxin-4 was reported to impair mediator release from both α-granules[70] and lysosomes[69], but not dense granules. However, in a separate study neutralization by SNAP-23 and syntaxin-7 antibodies impaired the secretion of platelet dense granules and lysosomes[69].

4.1.3.5 Macrophages

Macrophages express the R-SNAREs isoforms VAMP-2, -3 and -8 have been implicated in exocytosis of SV following activation of phagocytosis in murine macrophages which were mainly localized to recycling endosomes[71]. Those R-SNAREs were suggested to perform redundant roles and/or compensate for each other in phagocytic processes[34, 72]. VAMP-7 was identified on late endosomes[73]. Macrophages treated with TeNT showed impaired phagocytosis in murine J774 macrophages[74]. Clustering of VAMP-3 was found at the site of phagosome formation and was additionally incorporated into early phagosomes, suggesting its involvement in phagosomal maturation events[75]. Docking partners for macrophages include syntaxin-2, -3 and 4, all of which have been localized to both plasma membrane and isolated phagosomes[76]. Syntaxin-4 was recently identified as an important component involved in LPS-induced cytokine secretion in J774 macrophages[77].

4.1.4 Future directions

Human mast cells and basophils are the only cells that express the high affinity receptor for FcεRI and synthesize histamine. They are also an important sources of proinflammatory cytokines. In this study, which investigated the potential function of SNARE proteins, the precise role of those proteins in exocytosis remains to be determined. There are several techniques, which would facilitate the further characterization of trafficking events in human basophils.

Subcellular fractionation:

To discriminate different populations of granules, fractions from a sufficient number of human basophils (5-7 x 10^7) can be separated on a gradient and subjected to electrophoretic separation and immunoblot analysis for SNARE-specific antibodies, such as SNAP-23, syntaxin-4, and VAMP-7 and VAMP-8. Fractions may also be examined for granule-specific markers, CD63, β-hex and CLC protein. Previous studies on human eosinophils[7, 78] showed this is an effective and reliable technique.

Confocal microscopy:

Resting and activated human basophils can be double stained (granule-marker and SNARE-specific antibody) to test the intacellular mobilization of granules. One can argue the merit of such an approach; however, it can extend our knowledge on trafficking events in human basophils. Most of the current knowledge on human basophil biology came from electron microscopy. Live cell microscopy is another option to determine degranulation in human basophils.

Transfection of KU-812 with constructs contains SNARE proteins:

We have recently designed a GFP-syntaxin-4 construct in our lab. We fused a syntaxin-4 gene to a GFP (Green Fluorescent Protein) gene, in frame. The next step will be to transfer this construct to an inducible lentiviral system (pLenti-6), which means that it will only work if tetracycline is added to the medium. Therefore, we can control when the protein is expressed in the cell. Virus was generated by Dr. Wole Odemuyiwa, Post Doctoral Fellow in the Moqdel lab. Generated virus will be harvested from supernatant of the producer cell line (293T). This supernatant, containing the virus harbouring GFP-syntaxin-4, will be used to transfect KU-812. Preliminary data in our lab indicated that KU-812 was amenable for transfection. Following infection of the target cell, the virus integrates into the genome of the cell and becomes part of the cell. To overexpress the desired protein, syntaxin-4 (which will compete with the one the cell originally has) we will add tetracycline to induce GFP-syntaxin-4 expression. Because GFP emits green light when it is excited by the argon laser on the confocal microscope it is easy to detect whether KU-812 is producing GFP-syntaxin-4 or not. This overexpression system can be used to inhibit endogenous syntaxin-4. Using this approach, the precise role of each SNARE molecule can be determined in basophil mediator secretion and exocytosis.

Development of SNARE-deficient mouse model

The detrimental effect of inflammatory cells is postulated to be due to the release of proinflammatory mediators. Potentially, availability of SNARE-deficient mice will facilitate the research on the effect of granulocytes in granule/vesicle stores and mediator release in pulmonary inflammation. The mutant SNAP-25-deficient mouse model was previously described in the literature[79]. Homozygotes did not survive beyond gestation day 6, but heterozygotes have a normal life-span but express reduced levels of SNAP-25 mRNA and protein in the brain[79]. However, upregulation of SNAP-25 has been shown to preserve vital neuromuscular function. Similarly, macrophages derived from VAMP-3 knockout mice did not exhibit impaired phagocytosis of phagosome maturation[80]. Our study suggests that VAMP-7 is critically involved in basophil exocytosis. In future, design of VAMP-7- deficient mice may shed light on the critical role of this protein in granule/vesicle trafficking in human basophils.

4.1.5 Conclusions

My project is an integral part of the translational research of exocytosis in allergic inflammation and asthma. Exocytosis is a critical event in the activation of inflammatory cells. Our focus of interest involved a relatively rare inflammatory cell namely human basophils. Because activation of this cell, as well as MC leads to the release of potent mediators, they are directly implicated in the exacerbation of allergic inflammation including contact urticaria, allergic rhinitis, antagonism of asthma and anaphylactic reactions. The novelty of the current project is the identification of proteins which appear to be critical in exocytosis. Our data suggests that VAMP-7 plays an important role in distal events of exocytosis. Whether this structural uniqueness translates to functional differences *in vivo* remains to be determined. Determining functional versus redundant SNARE isoforms involved in secretory processes may prove to be an effective method to develop ways to impair mediator release from human basophils and MC. Our ultimate goal is to identify intacellular targets for potential clinical application and novel therapeutic strategies aimed at preventing and/or modulating the allergic response.

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Appendix

Table A-1. Additional table to Chapter 1: major differences between MC and human basophils.

Mast cells	**Basophils**
Differentiation and maturation completed in tissues, mature cells not found in blood	Differentiation in the bone marrow, present in blood, recruited to inflammatory sites
Numbers and phenotype are regulated by multiple microenvironment factors: SCF, IL-3, IL-4	Numbers are regulated primarily by IL-3
Can proliferate in tissues	Do not proliferate in tissues
Life span: months in tissue	Life span: 1-3 days
PGD_2, PGF_2, NO	Charcot-Leyden crystal protein and granule major basic protein
Strongly express SCF-R (c-kit/CD117)	Strong surface expression of IL-3α chain (CD123)

Materials

Buffered salt solution (BSS), pH 6.8:

	10X stock (500 ml)
137 mM NaCl	**40 g**
2.7 mM KCl	**1 g**
2 mM $MgCl_2$	**2 g**
20 mM PIPES	**3 g or 10 mL 1 M PIPES**

- Adjust pH of stock to 6.8 with 10 M NaOH (do this slowly if using powdered PIPES, as PIPES takes time to dissolve). Check pH of diluted solution before use.
- Dilute solution 1:10 before use (usually make up 100 ml for each assay) and add 1 mg/ml BSA.

EGTA solution (pH 6.8)
10 x 10^6 cells (10 ml) cell suspension/plate (density 1 x 10^6 cells/ml)
in 10% Triton-X-100 (in H_2O)
Streptolysin-O (100 µg/ml)
100 mM GTPγS (Boehringer Mannheim 95-97% pure solution)
Calcium buffers (*p*Ca 5 and 8) (made from protocol on previous page:
Inhibitory antibody (AZIDE FREE)
Isotype control antibody (AZIDE FREE)
Sigma TMB solution (T8540)
V-well 96-well microplates (volume/well = 0.25 ml)
Flat-well 96-well microplates (Falcon)
Remove from fridge:
    10% CHAPS (in H_2O)
    10% TX-100 (in H2O)

TMB solution (myeloperoxidase substrate) just before use

Remove from freezer:

100 mM ATP (may be frozen and thawed repeatedly)

Streptolysin-O (100 μg/ml) **(do not repeatedly freeze and thaw)**

Calcium buffers (pCa's can be frozen and thawed repeatedly)

100 mM GTPγS (may be frozen and thawed repeatedly)

Antibody (isotype + test)

Method

- Prepare 100 μM GTPγS by adding 1 μl frozen stock (100 mM) to 0.999 ml BSS buffer. Leave on ice.
- For KU-812 prepare 1% TX-100 similarly (as for eosinophil assay – Mike Logan PhD thesis). Leave on ice.
- Make up two tubes of pCa 5 and pCa 8 solutions:
  - **pCa 5** tube: Add 1.733 ml BSS, 900 μl GTPγS stock, 277 μl pCa 5, and 90 μl ATP (100 mM). Leave on ice.
  - **pCa 8** tube: Add 2.633 ml BSS, 277 μl pCa 8, and 90 μl ATP (100 mM). Leave on ice.
- Prepare 2.0 μg/ml SLO solution. Leave on ice.
- Prepare inhibitory antibody to a final concentration of 2 μg/ml in either 0.2 μg/ml SLO. Leave this on ice.
- Prepare inhibitor-SLO solutions to go into microplate by sequential dilution using the LOGSTEPS program. For example, for VAMP-2 mAb (1 mg/ml stock) :

Tube #	Antibody	SLO (μl of 0.2 μg/ml)	Ab conc with cells (diluted 2X)
1	138.6 μl of #2	160 μl	0.10 μg/ml
2	138.6 μl of #3	160 μl	0.22 μg/ml
3	138.6 μl of #4	160 μl	0.46 μg/ml
4	138.6 μl of #5	160 μl	1.00 μg/ml

5	138.6 μl of #6	160 μl	2.15 μg/ml
6	138.6 μl of #7	160 μl	4.64 μg/ml
7	4.4 μl of 1 mg/ml anti-VAMP-2 or 8.8 μl of 0.5 mg/ml isotype	435.6 μl or 431.2 μl	10.00 μg/ml

Total volume in each tube = 160 μl except for tube #6 = 440 μl

- Make up isotype control by using same series of antibody dilutions in SLO.

Microplate preparation

While spinning down 5 x 10^6 cells and resuspending these in 5 ml BSS, use a V-welled microtitre plate on ice to pipette following reagents.

- Add 50 μl of appropriate inhibitor-SLO mix into each well except Controls in Column 10, Blanks in Column 11 and Lysed Cells in Column 12.
- For **Columns 10 and 11** on the plate, add 50 μl SLO.
- For **Column 12** on the plate, add 50 μl 1% CHAPS in BSS.
- Resuspend cells in BSS at 1 x 10^6/ml. Do not leave cells in BSS any longer than 5-10 min as they gradually lose their secretory capacity.
- Remove pCa 5 and pCa 8 solutions from ice and allow these to warm at room temperature.
- Remove plate from ice and allow this to warm at room temperature.
- Get a reagent tray and adjust a multichannel pipette to 50 μl/well.
- Chill down some BSS to get it ready to stop the reaction.
- Pour cells into a reagent tray and quickly transfer 50 μl cells to all wells. Mix cells briefly to ensure mixing of SLO and inhibitor with cells. When cells are added to **Column 12** on the plate, make sure that you pipette the mixture up and down 3-4 times to assist in complete solubilization of cells.

- Place microplate into 37°C incubator for 2 min. The cells will be permeabilized in this step, so do not run over time!
- Pour pCa 5 and pCa 8 into different reagent trays.
- Remove microplate from incubator at 37°C at 2 min and transfer 50 µl of **pCa 5** into all wells EXCEPT Columns 11 and 12.
- Transfer 50 µl of **pCa 8** into Columns 11 and 12.
- Incubate for 10 min at 37°C.
- Stop reaction by adding 100 µl ice-cold BSS to each well
- Centrifuge plate for 5 min at 1500 rpm (~350g) at 4°C
- Remove supernatants for analysis of secreted mediators (TX-100 lysed represent total granule-derived proteins)

Secretion assay plate layout:

	1 Control #1 ↓	2 Control #2 ↓	3 Control #3 ↓	4	5 Inhibitor #1 ↓	6 Inhibitor #2 ↓	7 Inhibitor #3 ↓	8	9	10 Control (no Ab) ↓	11 Blank (pCa 8) ↓	12 0.5% CHAPS Lysed cells ↓
A	0.1 µg/ml →											
B	0.22 µg/ml →											
C	0.46 µg/ml →											
D	1 µg/ml →											
E	2.15 µg/ml →											
F	4.64 µg/ml →											
G	10 µg/ml →											
H	Empty →											